Universidade de Aveiro Departamento de Química
Ano 2012

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Fármacos epigenéticos regulam níveis de H2A.Z via sobrexpressão do gene *SIRT1*

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Epi-drugs regulate H2A.Z via *SIRT1* upregulation in prostate cancer

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica da Professora Doutora Carmen Jerónimo, Instituto Português de Oncologia do Porto e Departamento de Patologia e Imunologia Molecular do Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, e do Professor Doutor Francisco Amado, Departamento de Química da Universidade de Aveiro

o júri

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palavras-chave Cancro da Próstata, Fármacos Epigenéticos, H2A.Z, Sirtuina 1

Resumo

As actuais abordagens terapêuticas para cancro de próstata (PCa) avançado são limitadas e ineficazes. Uma vez que as aberrações epigenéticas são muito comuns em PCa, o uso terapêutico de fármacos epigenéticos pode ser uma alternativa viável. A variante de histona H2A.Z e sua forma acetilada têm sido associadas com a activação de oncogenes em células de PCa, devido à sua localização perto do TSS. Assim, objetivou-se avaliar o efeito de fármacos epigenéticos na regulação da expressão da variante H2A.Z.

Três linhas celulares de PCa (LNCaP, DU145 e PC-3) foram tratadas com 5-aza-2'-desoxicitidina (DAC) e tricostatina A (TSA). Os níveis de transcripto e proteína dos genes *H2AFZ* e *SIRT1* foram avaliados por qRT-PCR e Western Blot, respectivamente. ChIP foi realizado para avaliar em que medida o tratamento alterava o padrão de marcas de histonas. Subsequentemente, a exposição a nicotinamida e resveratrol, um inibidor e um indutor de actividade sirtuina 1, respectivamente, foi realizada para avaliar o papel da sirtuina 1 nos níveis H2A.Z. Além disso, um ensaio de ligação proximal (PLA) foi realizado para avaliar a capacidade dos farmacos epigenéticos em regular a interacção entre a sirtuina 1 e a H2A.Z.

O tratamento com TSA, sozinho ou combinado com DAC, levou a um aumento dos níveis de transcripto do gene *H2AFZ*, embora com uma simultânea diminuição dos níveis de proteína. Por outro lado, os níveis de transcripto e de proteína do gene *SIRT1* aumentaram após a exposição aos fármacos. O ChIP revelou um aumento de marcas de activação da transcrição na região do TSS para ambos os genes. Surpreendentemente, após a inibição da sirtuina 1, níveis H2A.Z aumentaram abruptamente, enquanto que a indução da sirtuina 1 levou a uma queda abrupta nos níveis de H2A.Z. Finalmente, os resultados de PLA mostraram que os fármacos epigenéticos favorecem a interacção entre as duas proteínas estudadas.

Embora os fármacos epigenéticos sejam capazes de regular a transcrição de ambos os genes, a falta de correlação entre os níveis de mRNA e proteína para H2A.Z sugerem o envolvimento de um mecanismo pós-translacional. De facto, a sirtuina 1 tem a capacidade de degradar H2A.Z, tal como previamente descrito para cardiomiócitos. Desta forma, os níveis de proteína H2A.Z são indirectamente regulados por fármacos epigenéticos, provavelmente através da sobreregulação do gene *SIRT1*. Em tumores primários da próstata, a sobreexpressão de *H2AFZ* e subexpressão de I validam os dois genes como alvos de tratamento epigenético.

keywords Prostate cancer, Epigenetics Drugs, H2A.Z, Sirtuin 1

abstract

Current therapeutic approaches for advanced prostate cancer (PCa) are limited and mostly ineffective. Because epigenetic aberrations are common in PCa, the therapeutic use of epigenetic modulating drugs might provide a therapeutic alternative. The histone variant H2A.Z and its acetylated form have been previously associated with activation of oncogenes owing to their location near the TSS in PCa cells. Thus, we aimed to evaluate the effect of epigenetic modulating drugs on H2A.Z expression.

Three PCa cell lines (LNCaP, DU145 and PC-3) were treated with 5-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA). Transcript and protein levels of *H2AFZ* and *SIRT1* were assessed by quantitative RT-PCR and Western blot, respectively. ChIP was performed to evaluate how treatment altered the pattern of histone marks. Subsequently, exposure to nicotinamide and resveratrol, an inhibitor and an inducer of sirtuin 1 activity, respectively, was performed to evaluate the role of sirtuin 1 in H2A.Z levels. In addition, PLA was performed to assess whether epigenetic drugs regulate sirtuin 1 and H2A.Z interaction.

Treatment with TSA alone and combined with DAC led to an increase of *H2AFZ* transcript levels, although with a concomitant decrease in protein levels. Conversely, *SIRT1* transcript and protein levels increased after drug exposure. ChIP revealed an increase of activation marks within the TSS region for both genes. Remarkably, after inhibition of sirtuin 1, H2A.Z levels increased dramatically, whereas induction of sirtuin 1 led to an abrupt decrease in H2A.Z levels. Finally, PLA assay results showed that epigenetic modulating drugs favored the interaction between sirtuin 1 and H2A.Z.

Although epigenetic drugs are able to regulate both *H2AFZ* and *SIRT1* transcription levels, the lack of correlation between mRNA and protein levels for H2AZ suggest the involvement of a post-translational mechanism. Indeed, sirtuin 1 has the ability to degrade H2A.Z, as previously reported for cardiomyocytes. Thus, H2A.Z protein levels are indirectly regulated by epigenetic drugs, probably through *SIRT1* upregulation. In primary PCa, overexpression of *H2AFZ* and downregulation of *SIRT1* validate both genes as targets of epigenetic treatment.

TABLE OF CONTENTS

FIGURES LIST

Figure 10 – Statistics concerning PCa incidence: **A** – worldwide incidence of PCa **B** – incidence of cancer in Portugal, showing PCa is the most frequent neoplasia; **C** – incidence of cancer worldwide, showing that PCa is only surpassed by lung cancer as the most frequent neoplasia in males (Globocan, 2010).

Figure 11 – Gleason score for histopathological grading of PCa, considering that differentiation decreases from 1 to 5 (adapted from Epstein *et al*, 2011).

Figure 12 – Model of gene transcription regulation dependent of H2A.Z occupancy in PCa. **(A)** H2A.Z, by some uncertain mechanism, become hypoacetylated or is replaced by the uncetylated form of H2A.Z, leading to decreased expression of tumor suppressor genes. **(B)** Nearby the TSS of oncogenes, after neoplastic transformation, H2A.Z become acetylated or is replaced by its acetylated form, promoting the expression of these genes (Valdés-Mora *et al.*, 2012).

Figure 13 – Representation of the mechanism of action of 5-aza-2'-deoxycytidine, which is incorporated during DNA synthesis, replacing the standard cytidine (adapted from Fandy *et al.*, 2009).

Figure 14 – TSA inhibits the activity of HDAC, allowing the histones to remain acetylated, bringing the chromatin into a transcriptional active state (adapted from Yoshida, 2008).

Figure 15 – Representation of how TSA interacts with the catalytic site of a HDAC homologue, forming a complex and inhibiting the catalytic capacity of the enzyme (Yoshida *et al.*, 2001).

Figure 16 – Transcript levels of *H2AFZ* in three distinct cell lines (**A-** LNCaP, **B-** PC-3 and **C-** DU145) after exposure to DAC and/or TSA. The results are presented as fold variation in comparison to the control experiment.

Figure 17 – Results for **(A)** Western Blot and **(B)** fold variation of optical density for H2A.Z protein levels in **(1)** LNCaP, **(2)** PC-3 and **(3)** DU145 cell lines. Results were normalized using the constitutive protein β-actin and are displayed as fold variation in comparison with *Mock* (control).

LIST OF TABLES

ABREVIATURES

AcH2A.Z – acetylated H2A.Z

AcH3 – acetylated H3

APS – ammonium persulfate

AR – androgen receptor

AS – active surveillance

Bcl_2 – B-cell lymphoma 2 protein

BPH – benign prostate hyperplasia

BRCA1 –breast cancer type 1 susceptibility protein

CD44 – cell-surface glycoprotein; CD44 antigen

CDK8 – gene that encodes for cyclin-dependent kinase 8

ChIP – chromatin immunoprecipitation

DAC – 5-aza-2'-deoxycytidine

DNA – deoxyribonucleic acid

DNMTi – DNA methyltransferases inhibitors

DNMTs – DNA methyltransferases

ER – estrogen receptor

FRET – fluorescence resonance energy transfer technique

gDNA – genomic deoxyribonucleic acid

H3K4me – mono-methylated histone H3 at lysine 4

H3K4m2 – di-methylated histone H3 at lysine 4

H3K4me3 – tri-methylated histone H3 at lysine 4

H3K9me – mono-methylated histone H3 at lysine 9

H3K9me2 – di-methylated histone H3 at lysine 9

H3K9me3 – tri-methylated histone H3 at lysine 9

H3K18ac – acetylated histone H3 at lysine 18

H3k27me3 – tri-methylated histone H3 at lysine 27

HATs – histones acetylases

HDACi – histones deacetylases inhibitors

HDACs – histones deacetylases

HDMs – histones desmetilases

HMTs – histones methyltransferases

Lys – lysine

m5C – 5-methylcytosine

MH2A – macroH2A

mRNA – messenger ribonucleic acid

NAD^+ – nicotinamide adenine dinucleotide

NIC – nicotinamide

NPT – normal prostate tissue

PCa – prostate cancer

PCR – polymerase chain reaction

PIN – prostate intraepithelial neoplasia

PSA – prostate-specific antigen

PTM – posttranslational modification

qRT-PCR – quantitative reverse transcriptase polymerase chain reaction

RES – resveratrol

RIPA – radio immune precipitation assay

RNA – ribonucleic acid

SAM – S-adenosyl-methionine

TEMED – tetramethylethylenediamine

TSA – trichostatin A

TSS – transcription start site

WW – watchful waiting

I
INTRODUCTION

I - INTRODUCTION

Chromatin is the physiological template of human genome. Its basic unit – the nucleosome core particle (*Figure 1*) – consists of 146 DNA base pairs organized around an octamer consisting of two copies of each highly conserved core histone protein – H2A, H2B, H3 and H4 [1].

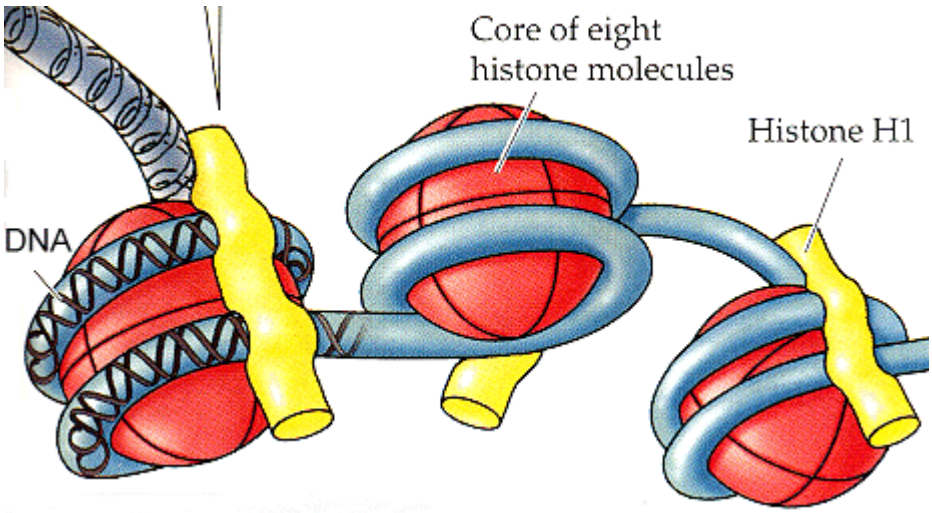

Figure 1 – Schematic representation of a nucleosome (Purves *et al.*, 1997 [2]).

Dynamic modulation of chromatin structure (chromatin remodeling), is a key component in the regulation of gene expression, DNA replication and repair and chromosome condensation and segregation [3]. Disruption of these processes is intimately associated with human diseases, including cancer. Hence, the comprehension of mechanisms that tightly regulate gene expression in cancer, such as the epigenetic mechanisms, not only allows the development of better diagnostic and prognostic markers, but most important, the development of therapeutic approaches that might contribute for cancer abolition [4].

Particularly, information regarding the role of histone variants in prostate neoplasm is rather poorly understood, as well as the effects of epigenetic treatment with histone deacetylases inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) in chromatin remodeling and histone variants deposition.

II
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II – STATE OF THE ART

1. Epigenetics

The concept of epigenetics was firstly introduced by Conrad Waddington, who defined epigenetic as "the branch of biology which studies the casual interactions between genes and their products which bring the phenotype into being" [5, 6]. But the original definition has been changed all over the years, being nowadays defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [7, 8]. The epigenetic mechanisms that are responsible for chromatin remodeling can be divided into four different groups: DNA methylation, covalent histone modifications or posttranslational modifications of histones, non-covalent modifications of histones, such as incorporation of non-canonical histones and nucleosome remodeling, and non-coding RNAs, such as microRNAs [5].

2. Epigenetic mechanisms and their deregulation in cancer

2.1 DNA methylation

The methylation of DNA is probably the most studied epigenetic mechanism. This type of modification plays an important role in gene expression regulation, since it provides a stable gene silencing. In mammals, this covalent modification of DNA usually occurs at cytosines followed by guanine, known as CpG dinucleotides [9]. Interestingly, CpG dinucleotides are not evenly distributed in the genome, but, instead they are mostly found in 'CpG islands', which occupy around 60% of human gene promoters, and in regions of large repetitive sequences [10].

In one hand, whilst most of the CpG sites in genome are methylated, the majority of CpG islands usually remain unmethylated during development and in differentiated tissues [11]. However, some CpG island promoters become methylated during development, resulting in long term transcriptional silencing, such as in X-chromossome (X-chromossome inactivation) and in imprinted genes [9]. On the other hand, CpG dinucleotides that are found in repetitive sequences genome are heavily methylated, in

order to inhibit chromosomal instability, since non-coding DNA and transposable DNA elements are silenced [11].

The mechanism of methylation of DNA is well established (*Figure 2*). The reaction is catalysed by DNA methyltransferases (DNMTs) and involves the use of a cosubstract, S-adenosyl-methionine (SAM), which functions as the donor of the methyl (-CH3) group. This reaction culminates in the formation of a new DNA base, 5-methylcytosine, which is nothing more than a cytosine methylated in the carbon 5 [12, 13].

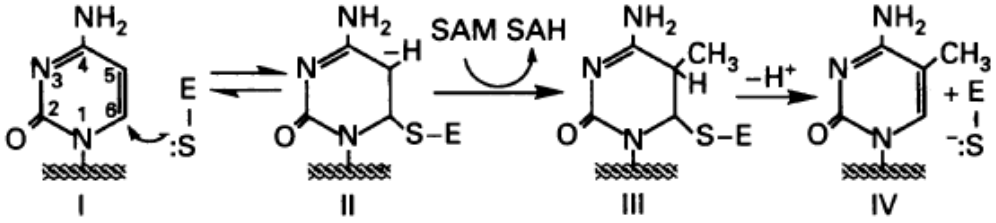


Figure 2 – Mechanism of cytosine methylation: reaction catalysed by DNMTs, represented as ES, using SAM as the methyl donor group (Gabbara *et al.*, 1995 [14]).

Usually, a cancer epigenome is characterized by global hypomethylation and site-specific CpG island promoter hypermethylation, being both events important in carcinogenesis [4].

DNA hypomethylation occurs in several sites of genome, such as repetitive elements, retrotransposons and gene deserts, leading to genomic instability, due to chromosomal rearrangements and retrotransposons translocation to other genomic areas [15, 16]. Simultaneously, another consequence of hypomethylation, is loss of imprinting. *IGF2* gene is an example where loss of imprinting results in the biallelic expression of this important growth factor [17].

Similarly, while there is a general hypomethylation on genome, there is a site specific hypermethylation that contributes to tumor formation, since it leads to tumor suppressor gene silencing, thus serving as the second event of tumor initiation in the Knudson's two-hit model [4].

2.2 Post-translational modifications of histones: the histone code

Histones are among the slowest evolving proteins known and they are a major component of chromatin, the protein–DNA complex fundamental to genome packaging, function, and regulation. Histone proteins contain a globular C-terminal domain and a N-terminal tail, being the latter very susceptible to posttranslational modifications (PTMs). This is due to the nature of the aminoacids sequence of histones, since there are several sites where they can undergo covalent modifications [18]. More than 60 residues have been reposted to harbour PTMs, specifically lysine (*Figure 3*) [19]. Even though the most understood PTMs are acetylation and methylation, other covalent transformations might occur such as phosphorylation, ubiquitylation and sumoylation. Alteration of the pattern of histone modifications controls important cellular processes, such as transcription, replication and DNA repair [18].

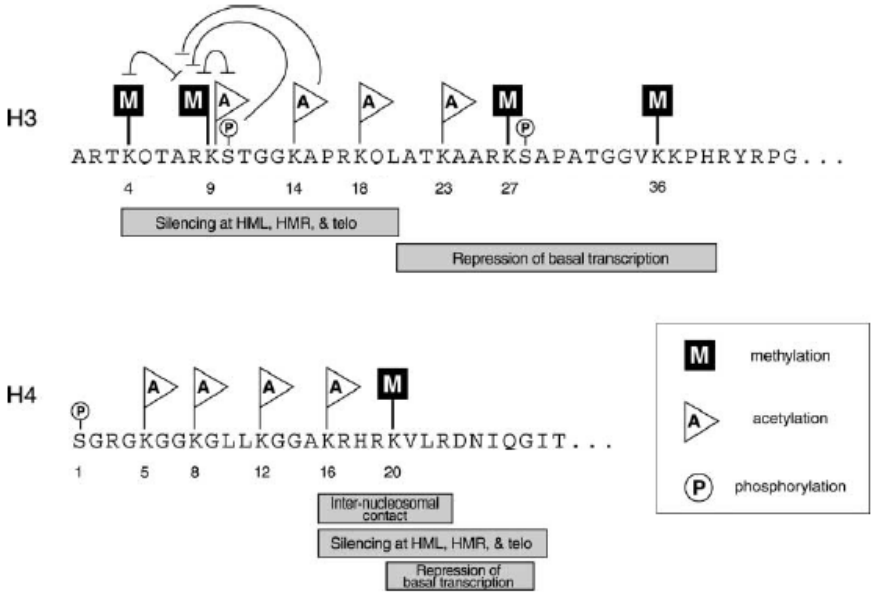


Figure 3 – Most frequent covalent modifications in human histones H3 and H4 (adapted from Richards *et al.*, 2009 [20])

As previously mentioned, an important covalent modification of histones is acetylation, modulating chromatin structure and nucleosome packing, exposing or hiding specific genes [21]. Since histones contain large amounts of basic amino acids (lysine and arginine), acetylation neutralizes the abundance of positive charges, decreasing histone affinity for the phosphate backbone of DNA [18, 21]. In general, histone acetylation results in charge neutralization and separation of DNA from the histones, allowing nucleossomal

DNA to become more accessible to transcription factors. Hence, histone hyperacetylation has been correlated to transcriptionally active regions of the genome, whereas silent regions are hypoacetylated (*Figure 4*) [18, 22]. The dynamic and reversible equilibrium between acetylated and deacetylated states of histones are regulated by two main protein groups: histone acetyltransferases (HATs) and histone deacetylases (HDACs).

Methylation is a PTM commonly found on histones and, as the acetylation, this type of histone modification has a significant role in chromatin remodeling. Namely, methylation may be a mechanism to prevent acetylation on lysine residues [23]. The enzymes responsible for these modifications are histones methyltransferases (HMTs) and its effect is reversed by histones demethylases (HDMs), recently discovered [24, 25]. As same as DNMTs, HMTs uses SAM as the methyl group donor, and, contrasting with histone acetyltransferases, histones methyltransferases exhibit higher substract specificity [24].

Unlike acetylation, which is associated with gene transcription activation, methylation of histones might lead to activation or repression of specific genes depending on the specific residue. For instance, trimethylation of lysine 4 (H3K4me3) and methylation of lysine 36 and lysine 79 on histone 3 (H3K36me and H3K79me) are found in the promoters of transcriptionally active genes. However, methylation of lysine 20 of histone 4 (H4K20me) and trimethylation of lysine 9 and lysine 27 on histone 3 (H3K9me3 and H3K27me3, respectively) are enriched in promoters of transcriptionally silenced genes. In fact, the latter two histone marks are both associated to gene silencing in mammalian cells [18].

Noticeably, the equilibrium between HMTs and HDMs has an important role in the overall histone methylation profile. In fact, the equilibrium verified in a normal mammalian cell, is disrupted during disease [26].

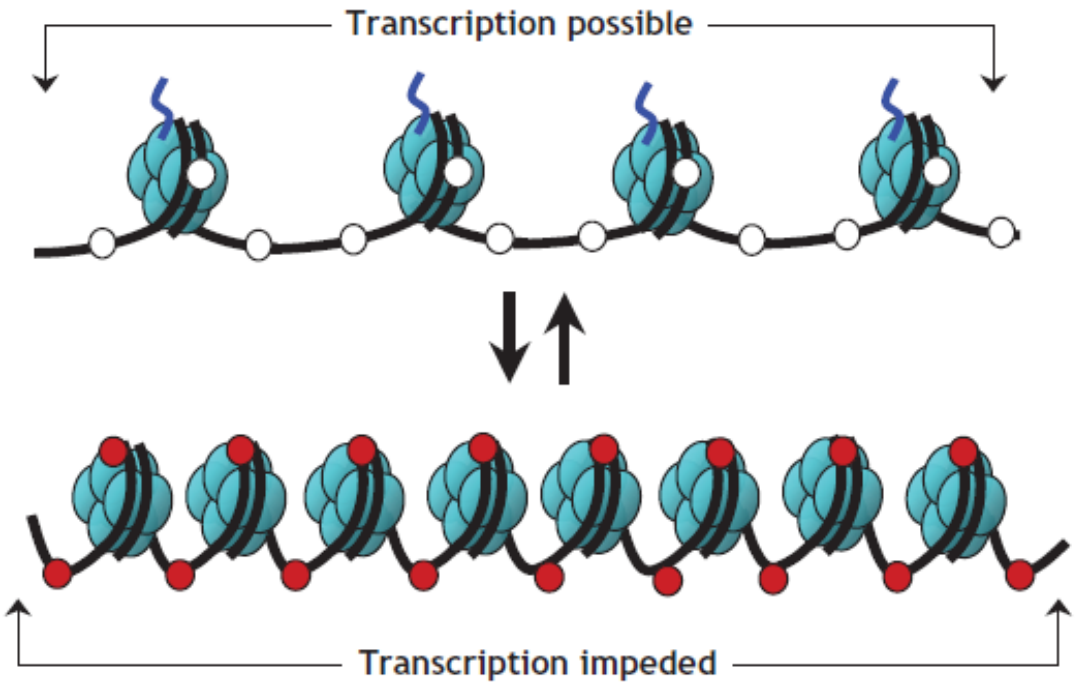


Figure 4 – Schematic representation of how histone acetylation works with DNA methylation in order to repress or activate transcription (acetylation indicated as the tails in the nucleosomes and methylation represented by the circles in DNA strand – red indicates an hypermethylated state, while white indicates a normal or hypomethylated state) (adapted from Rodenhiser *et al.*, 2006 [26]).

2.3 Histones variants and nucleosomal repositioning

In most organisms, there are multiple copies of the histone genes encoding for the major histone proteins. These genes are highly similar in sequence, expressed primarily during the S phase of the cell cycle and codify for the bulk of the cellular histones. Whilst histones are among the more conserved proteins known, there are nonallelic variants of the major histones that might have significant differences in primary sequence. Some variants have distinct biophysical characteristics that are thought to alter the properties of nucleosomes, while others are localized in specific regions of the genome. Additionally, while canonical histones genes are expressed only during the S phase of the cell cycle, variant histone genes, or non-canonical histone genes, are expressed through whole cell cycle [27-29].

Some variants replace with the canonical histones during development and differentiation, and are therefore referred to as replacement histones [30]. This replacement

often results in the variants becoming the predominant species in the differentiated cells [31, 32]. These observations have led to the suggestion that the histone variants have specialized functions regulating chromatin dynamics, as displayed in *Figure 5*.

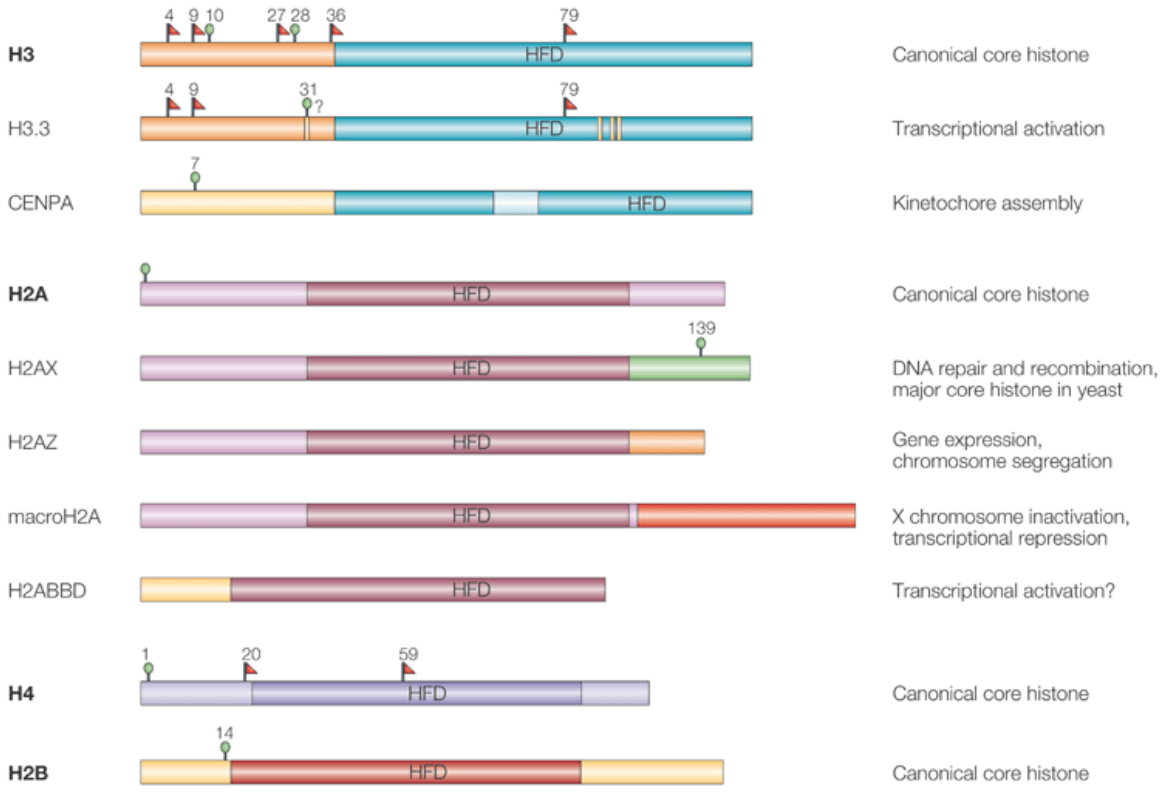


Figure 5 – Representative scheme of canonical and non-canonical histones, most relevant and well-established sites for lysine methylation and serine phosphorilation (red flags – methylated lysines; green circles – phosporilated serines). The specialized functions purposed for histone variants are as well indicated (Sarma *et al.*, 2005 [29]).

Some specific cases, such as H2A.Z and H3.3, histones variants of H2A and H3, respectively, are enriched in promoters of active genes and they might mediate nucleosome stability. In addition, H2A.Z can be inserted in nucleosomes in order to prevent DNA methylation, thus regulating gene activity [33, 34].

Regarding variant nucleosomes, one of the most relevant structural occurrences is that these nucleosomes have changes on the exposed surface, when compared with canonical nucleosomes. MacroH2A (mH2A) has an extensive C-terminal tail that probably extends away from the nucleosome and imparts an asymmetrical structure to the variant nucleosome which is important for transcriptional repression [35].

Although the overall structure of H2A.Z nucleosomes is similar to the major H2A structure, two of the most interesting differences are the presence of an extended acidic patch on the nucleosome surface and a novel divalent cation-binding pocket. These changes alter protein/nucleosome and nucleosome/nucleosome interactions, as well as the higher-order folding of the chromatin, and are important for H2A.Z function during development [36].

Using FRET assays was possible to observe that the overall binding of the H2A.Z/H2B dimer to the H3/H4 tetramer is somewhat stable [37]. Recent data propose that the CenH3/H4 tetramer is more dense and rigid than an H3/H4 tetramer and may also be more stable [38]. Indeed it is possible that the additional rigidity might help to resist the microtubule pulling forces at the centromere during mitosis, or might aid in the assembly of kinetochore proteins. Likewise, the mH2A nucleosomes may also be more stable, though additional biophysical studies will be required to fully understand the differences between the variant and major histone subtypes [39]. Also, the *in vivo* consequences of a more stable variant nucleosome scattered among the canonical nucleosomes are difficult to predict.

In contrast to the other variant nucleosomes, the H2ABbd nucleosome structure may be weaker. In the absence of DNA it is unable to form a stable histone octamer, and the H2ABbd nucleosome organizes only 118 bp of DNA rather than the 147 bp around the histone core [40]. While these nucleosomes are not very mobile, they are less stable and more accessible to transcription factors [40]. Therefore, it is likely that the structural alterations in the H2A-Bbd nucleosome lead to a weaker nucleosome structure that facilitates gene activation.

2.4 Sirtuin 1 and H2A.Z: from the connection to cancer to their putative interaction

2.4.1 The role of H2A.Z in carcinogenesis

The variant H2A.Z is highly conserved from yeast to man, since 90% of its primary sequence is preserved between different species and it shows only 60% homology with H2A [41]. H2A.Z is found in approximately 10% of mammalian and chicken nucleosomes,

participating in different biological processes such as DNA replication, chromosome segregation, heterochromatin condensation and gene transcription [42, 43].

However, the mechanism by which H2A.Z affects chromatin stability is still not fully understood. In fact, different studies reported distinct and controversial conclusions: some claim that H2A.Z nucleosomes are more stable [44], while others have observed that H2A.Z incorporation destabilizes nucleosome core particles [45].

A possible role for H2A.Z in cancer development was first reported by genome wide gene expression profiling by Dunican *et al.*, reporting overexpression of H2A.Z in microsatellite instable sporadic colorectal tumors [31]. Later, Zucchi *et al.* found that this histone variant was also overexpressed in the latest stages of breast cancer [46].

Rhodes *et al*. have found that 69 genes were overexpressed in undifferentiated cancers relative to well differentiated cancers, being *H2AFZ* one of those genes. Since H2A.Z plays a role in chromatin remodeling and in a broad spectrum of transcriptional regulations, it has been suggested that *H2AFZ* might play a critical role in maintaining the undifferentiated cellular state of high-grade cancers [47].

In fact, the best characterized cancer model regarding H2A.Z role in carcinogenesis is breast cancer. Hua *et al*. described that H2A.Z overexpression and lymph nodes metastasis were tightly correlated in primary breast tumors [48]. More recently, it has been shown that, not only H2A.Z is important in the regulation of ERα-dependent transcription and estrogen-specific cell proliferation, but also that H2A.Z expression promoted cellular proliferation even when estrogen levels are low and during tamoxifen treatment (the pro-proliferative effects of H2A.Z overrides the toxic effects of tamoxifen) [49].

The combination between H2A.Z expression and short overall patient survival suggested that this histone variant might be a biomarker not only of tumor aggressiveness, but, eventually, a cause of cancer phenotype [50].

Additionally, it was already proposed the H2A.Z might be an important indirect target for breast cancer therapy, through c-Myc regulation, which is known as an activator of H2A.Z expression (*Figure 6*) [51].

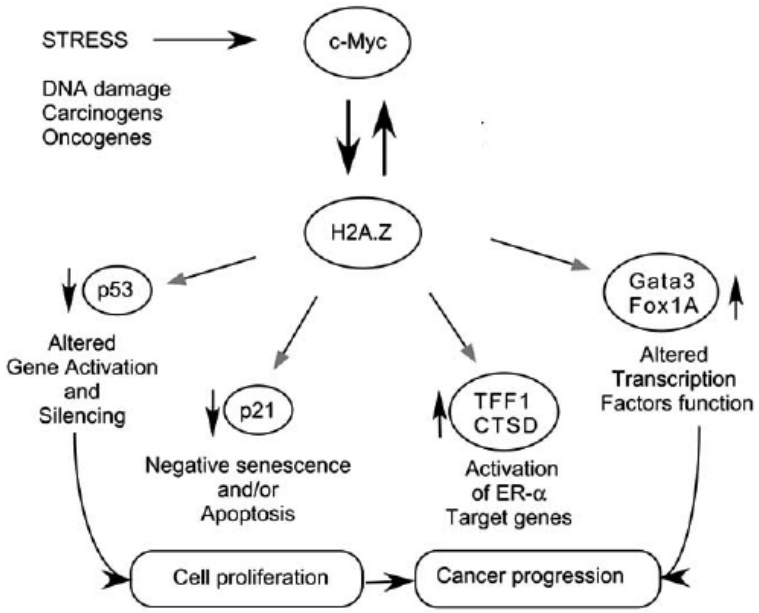


Figure 6 – Model of functional cooperation between H2A.Z and c-Myc in breast cancer. Upregulation of both proteins, leads to downregulation of *TP53* and *CDKN1A* and upregulation of estrogen target genes and transcription factors (adapted from Rangasamy *et al.*, 2010 [51]).

2.4.2 The dubious role of sirtuin 1

As previously mentioned, the equilibrium between the acetylated and deacetylated form of histones is tightly maintained by HATs and HDACs, respectively. However, during carcinogenesis, the action of these enzymes, mostly HDACs, are extremely altered [52].

HDACs are a group of 18 proteins which are divided into 5 different classes according to their homology (*Figure 7*) [53]. Class III HDACs, which entails sirtuins, are unrelated to the other classes, since they are dependent of NAD^+, and not Zn^{2+} ion like the others HDAC classes, being present in the nucleus (sirtuin 1, 6 and 7), cytoplasm (sirtuin 1) or mitochondria (sirtuin 3, 4 and 5) [54, 55]. Moreover, this specific HDAC class is not regulated by HDACi that block the activity of the other HDACs, such as trichostatin A (TSA) [56]. Instead, sirtuin 1 is inhibited by nicotinamide (NIC) [57] and its activity is enhanced by resveratrol (RES) [58].

Noticeable, several studies have suggested that some members of the HDAC class have a critical role in cancer. Interestingly, sirtuin 1, the most studied member of class III HDAC, has been reported as being critical in the neoplastic transformation. However, the results are quite controversial [59]. Some studies indicated *SIRT1* as an oncogene,

reporting its importance in p53 deacetylation, which ultimately leads to its inactivation [60]. Moreover, *SIRT1* has been found overexpressed in several cancer models, such as acute myeloid leukaemia [61] and primary colon cancer [62].

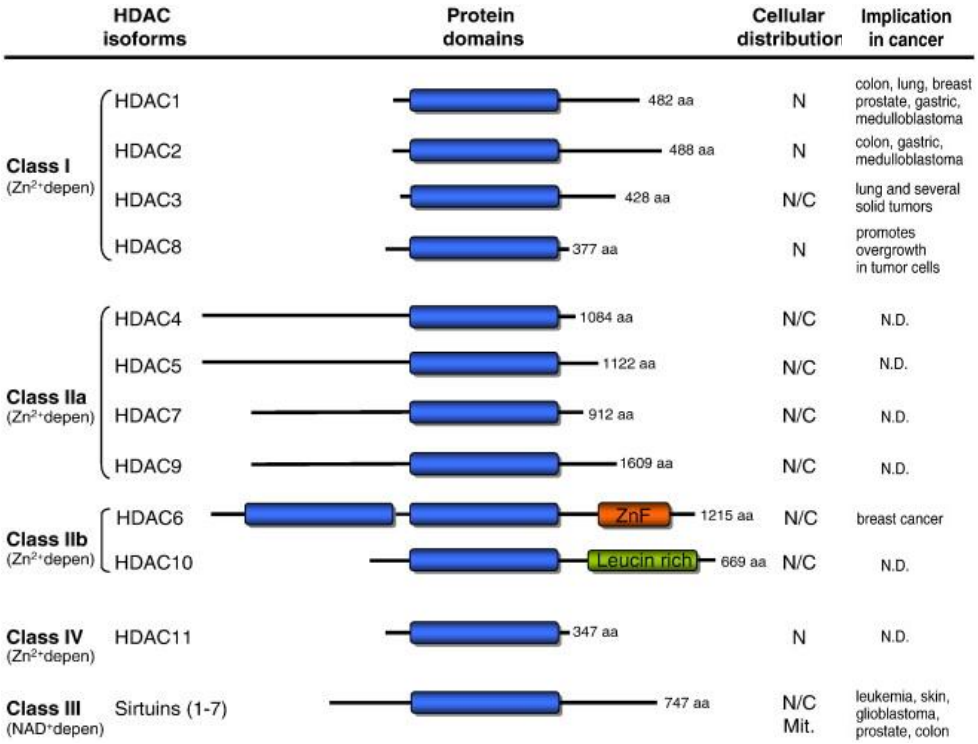


Figure 7 – Representative scheme of histone deacetylases classes, cellular location and implication in cancer (Di Marcotullio *et al, 2011* [63].

Contrarily, others studies refer *SIRT1* as an important tumor suppressor gene, being recently described as a haploinsufficient tumor suppressor, and has been found to be underxpressed in glioblastoma, bladder and ovarian cancers [64]. Interestingly, in prostate cancer (PCa) *SIRT1* has been reported both as being over and under expressed [64, 65]. Thus, it is yet unclear whether *SIRT1* acts as a tumor suppressor gene or an oncogene in this neoplasm.

2.4.3 Sirtuin 1 marks H2A.Z for degradation

Interestingly, an interaction between sirtuin 1 and the histone variant H2A.Z has been reported, that ultimately lead to degradation of the latter (*Figure 8*). Specifically, Chen *et al*. have found that sirtuin 1 deacetylated lysine 15 (Lys-15) of H2A.Z in cardiomyocytes, marking it for ubiquitylation. Hence, two specific residues – lysine 115

(Lys-115) and lysine 121 (Lys-121) – become ubiquitylated, being consequently degraded via an ubiquitin/proteasome-dependent pathway [66].

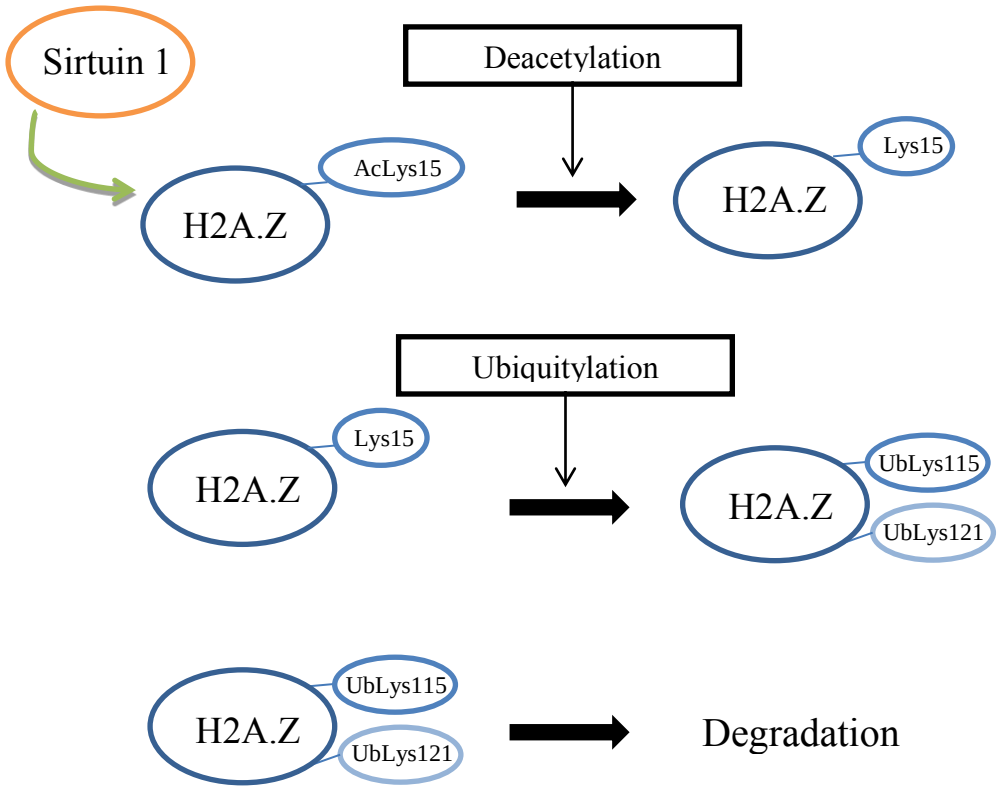


Figure 8 – Simplified representation of H2A.Z degradation via ubiquitin/proteasome-dependent pathway, initiated by sirtuin 1.

Remarkably, this event was only reported once and its confirmation in cancer models remains unknown. Nevertheless, if this mechanism is proven in cancer cells, this would support *SIRT1* as a tumor suppressor gene.

3. Prostate

Prostate is a gland about the size of a walnut and with a conical shape. Regarding its location, it is situated in the pelvic cavity, below the lower part of the symphysis pubis, above the superior fascia of the urogenital diaphragm, and in front of the rectum, through which it might be clearly felt, especially when enlarged [67]. The prostate is a gland belonging to the male reproductive system, being responsible for a hydrolytic secretion that constitutes about half the volume of the seminal fluid [68].

Although the concept of prostate anatomy has changed throughout the years, McNeal established the current and widely accepted division of prostate. Hence, McNeal definied that prostate is divided into four distinct zones: peripheral zone, central zone, transition zone and anterior fibromuscular stroma (*Figure 9*) [69, 70].

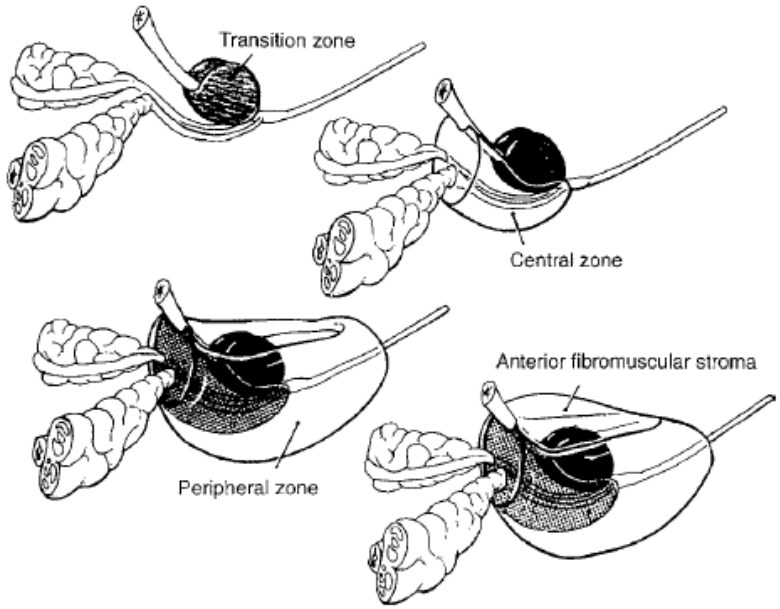


Figure 9 – Anatomy of normal prostate as described by McNeal (McNeal, 1980 [70]).

The peripheral zone includes all the prostatic glandular tissue at the apex as well as all of the tissue located posteriorly near the capsule. Interestingly, carcinoma, chronic prostatitis and postinflammatory atrophy are more probable to happen in this prostate zone than in the other three zones. The transition zone, which entails two similar portions of glandular tissue lateral to the urethra in the midgland, is where is observed the development of benign prostate hyperplasia (BPH) and, not that often, adenocarcinoma [71].

In addition to these different four zones, the prostate is involved by a capsule. In fact, the use of the term ‘capsule’ to define the fibrous layer that embraces the prostate, separating prostate stroma from extraprostatic fat fat tissue, is not truly correct. Despite the controversy, this terminology is still used until today [72].

3.1 From prostatic intraepithelial neoplasia (PIN) to PCa

Some studies concerning the histopathological evaluation of PCa tissue have conducted to the identification of a specific abnormality that is known to be the primary precursor lesion that might culminate in cancer [73]. Prostatic intraepithelial neoplasia, commonly known as PIN, is characterized as a continuum between low-grade and high-grade forms, with high-grade PIN being the immediate precursor of early invasive PCa [73].

The classification of high-grade PIN neoplasia as the precursor of cancer was achieved due to a different amount of evidences that support that idea. The location [74], appearance [75], chromosomal abnormalities [76-78], cell architecture [79] and differentiation markers [80, 81] are evidences pointing towards the notion that PIN lesions precedes PCa. Interestingly, PIN lesions do not produce high levels of prostate-specific antigen (PSA), thus not being identified in PSA screening test done in blood serum, but only in prostate biopsy samples [80].

A very relevant aspect of PIN lesions is their accurate identification by microscopy, when compared with other prostate pathological conditions, such as BPH and atypical adenomatous hyperplasia, which are not believed to be precursor states of PCa. In opposition, the lesion designated as proliferative inflammatory atrophy has been proposed as the precursor of PIN lesions [82].

3.2 Epidemiology of PCa: incidence, mortality and risk factors

PCa is the second most frequent neoplasia between male population worldwide (13.6% of the total), being only surpassed by lung cancer [83], and the number one neoplasia in the male population in developed countries (72.1% of the total cases of PCa are observed in developed countries) (*Figure 10*). Considering de overall population, male and female population, PCa is the fifth most frequent neoplasia in the world. In Portugal, PCa is the form of cancer with highest incidence between male population [83].

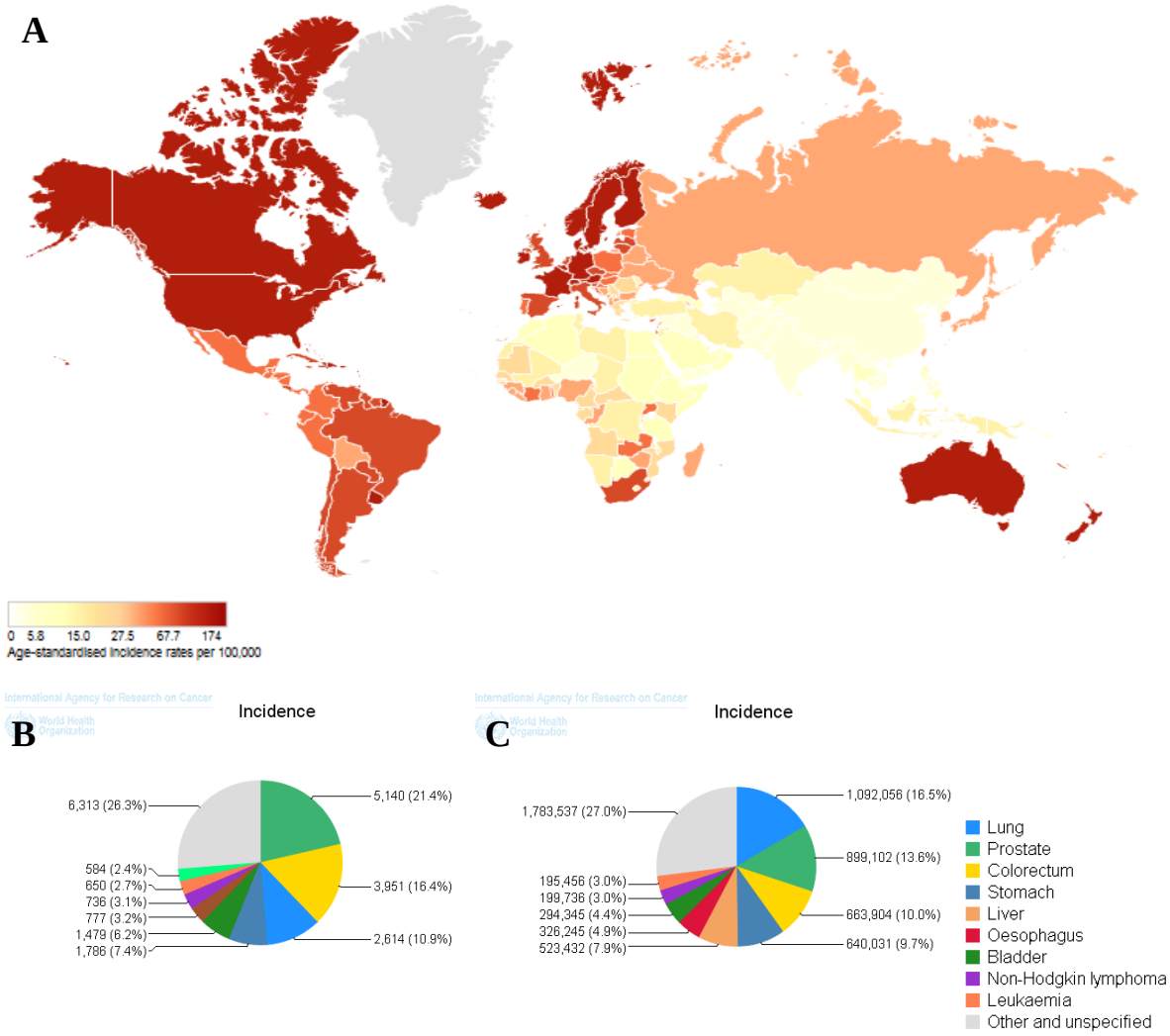


Figure 10 – Statistics concerning prostate cancer incidence: **A** – worldwide incidence of PCa; **B** – incidence of cancer in Portugal, showing PCa is the most frequent neoplasia; **C** – incidence of cancer worldwide, showing that PCa is only surpassed by lung cancer as the most frequent neoplasia (Globocan, 2010 [84]).

Not only there are differences between geographic areas affected by PCa, but there are also some changes which occurred throughout the years [85]. In United States of America, even though rates of PCa were stabilized until the 1980s, after the introduction of PSA screening, the rates of incidence of PCa increased 85% between 1987 and 1992 and, despite some subtle differences, the rates fairly remained stable [86].

That sudden increase in the number of PCa cases is mostly due to the introduction of serum PSA evaluation, which was described as the first method in the detection of prostate adenocarcinoma. Interestingly, after that, the stabilization of incidence rates occurred as a consequence of that new screening test, which possibly detected most of the

latent tumors in the population submitted to this evaluation [87]. It is important to mention that, despite the fact that the tendency observed in United States of America is verified in several countries, in Europe, the rates of PCa incidence are still increasing [83].

Regarding mortality, PCa is the sixth most mortal neoplasia among men, being responsible for 6.1% of cancer related deaths, excluding non-melanoma skin cancer. Contrarily to incidence rates, mortality rates have only changed slightly throughout the years [86]. The difference verified through the time can be explained by PSA screening, which, in one hand, may have led to an initial increase of mortality rates due to wrong death explanation (although deaths were attributed to PCa disease, in fact, people died from co-morbid conditions, such as cardiovascular disease or other neoplasm), and, on the other hand, the decreased observed in recent years may be due to an early diagnosis of the disease and hence a therapy applied on early stages of the neoplasia [87].

The risks for development of PCa are still very controversial, but three distinct risk factors are well established: increasing age, ethnicity and heredity. In fact, PCa is a disease directly related with aging, since incidence increases exponentially with age, being the peak of incidence on ages between 72 and 74 years [88].

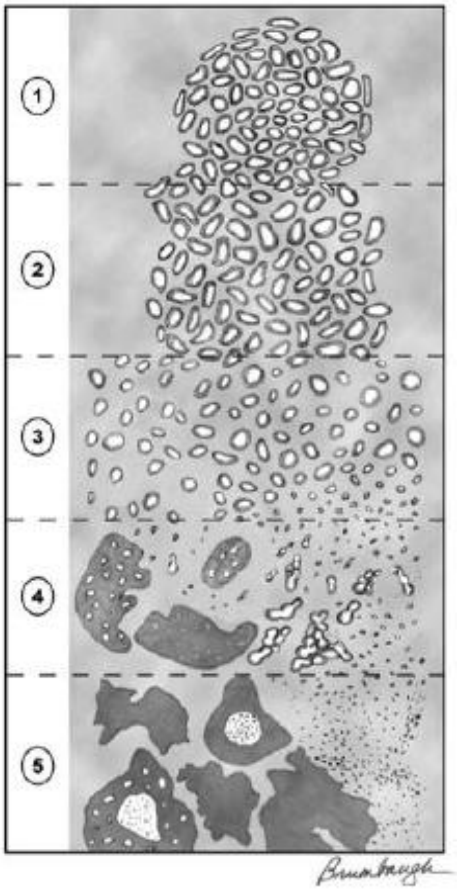
Regarding ethnicity, African Americans have the highest rates of PCa incidence and mortality, being followed by Caucasians, Hispanics and, finally, Asians and Pacific Islanders [89].

3.3 Histopathological granding and staging of PCa

Noticeably, the most prevalent form of PCa is adenocarminoma, accounting for about 95% of prostatic neoplasms. Actually, this neoplasia is silent, without specific symptoms, despite resembling some characteristics of BHP. Having that, and since the diagnosis of PCa is usually done in advanced stages, there is a need to evaluate tumor grading and staging [90].

The histologic grading by Gleason score is considered the strongest prognostic factor of patient's time to progression and the tumor is graded from 1 to 5 regarding only the architectural appearance under low magnification (*Figure 11*) [91]. On one hand, well differentiated PCa is characterized by a proliferation of microacinar structures lined by prostatic luminal cells without an accompanying basal cell layer and is scored from 1 to 2,

being considered the lowest grade of Gleason system. On the other hand, a solid pattern with central necrosis or infiltrating individual cells is considered the highest grade of Gleason system and is scored with 5 [92].



Legend:
1 – closely packed, small and uniform glands;
2 – more stroma between glands, that are round and oval, with minimal invasion of non-neoplastic tissue;
3 – irregular sized glands with angular shape and more infiltrative margins;
4 – fused, cribiform and poorly defined glands;
5 – occasional gland formation .

Figure 11 – Gleason score for histopathological grading of PCa, considering that differentiation decreases from 1 to 5 (adapted from Epstein *et al*, 2011 [93]).

However, considering the PCa heterogeneity, with two or more grades in the same tumor sample, Gleason chose to evaluate the prostatic neoplasm incorporating both primary and secondary grade into the system, being the first the most prevalent one. Hence, the Gleason scores possibilities range from 2 (1 + 1) to 10 (5 + 5) [94].

Concerning the staging for prostatic adenocarcinoma, in the early 1950s was implemented the staging system which is considered today [95]. It concerns the extent of primary tumor (T), regional lymph nodes status (N) and distant metastases (M).

Table 1 – Overview of International Union Against Cancer (UICC) TNM/pTNM staging system for PCa (adapted from Edge *et al.*, 2010 [96])

Stage	**Primary tumor, clinical (T)**
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor not palpable or visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within the prostate
T2a	Tumor involves half of one lobe or less
T2b	Tumor involves more than half of one lobe but not both
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicle(s)
	Primary tumor, pathologic (pT)
pT2[c]	Organ confined
pT2a	Unilateral, involving half of one lobe or less
pT2b	Unilateral, involving more than half of one lobe but not both lobes
pT2c	Bilateral
pT3	Extraprostatic extension
pT3a	Extraprostatic extension
pT3b	Seminal vesicle invasion
pT4	Invasion of bladder, rectum
	Regional lymph nodes (N)
NX	Regional lymph nodes cannot be assessed
N0	Noregional lymph nodes metastasis
N1	Metatasis in regional lymph node or nodes
	Distant metastases (M)
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s)

This classification allows a more precise stratification of patients into groups with distinct prognosis.

Regarding metastases, although these might occur in several areas, like kidney, breast, liver and brain, they are mainly observed into pelvic lymph nodes, bones and lungs [97]. Remarkably, the most used PCa cell lines, an *in vitro* model to study and understand PCa, results from PCa metastasis. For instance, LNCaP, an androgen-sensitive PCa cell line, is derived from the left supraclavicular lymph node metastasis, and PC-3, an androgen-insensitive PCa cell line, is derived from bone metastasis [98].

3.4 Prostate cancer treatment

The therapeutic decision for PCa is currently based in a multidisciplinary approach and takes into account the TNM classification and Gleason Score of the tumor, preoperative serum PSA level and patient's age, life expectancy, comorbidity and quality of life and it is based.

3.4.1 Treatment of clinically localized, early-stage disease

Two firstly considered therapeutic options are watchful waiting (WW) and active surveillance (AS), being subtly different. In the WW approach there is a setback in active treatment in patients who are not candidates for an aggressive local therapy, until a patient develops evidence of symptomatic disease progression, at which point androgen-deprivation treatment in initiated [99]. On the other hand, AS is more suitable for those patients who a curative option might be also offered. The patients with low risk of PCa that fulfill the requirements for AS therapy (*Table 2*) are initially not treated, but are followed and treated with a curative intent if progression occurs during follow-up, with regular serum PSA evaluations and periodic prostatic biopsies [100].

The removal of the whole prostate gland and the seminal vesicles – radical prostatectomy – is the only treatment that revealed a cancer-specific survival higher than WW or AS [101]. Nowadays, this surgical approach is so polished that it offers high rates of cure and concomitant decreased morbidities, such as erectile dysfunction and urinary incontinence [96].

Table 2 – Eligibility criteria for potential AS patients (adapted from Heidenreich *et al*., 2011 [102])

	Criteria
Active Surveillance Approach	Clinically confined prostate cancer (T1-T2)
	Gleason score ≤ 6
	Three or fewer biopsies involved with cancer
	≤ 50% of each biopsy involved with cancer
	PSA ≤ 10ng/mL

Alternatively to radical prostatectomy, external-beam radiation therapy [103] and transperineal low-dose rate brachytherapy [104] may be offered. In fact, both approaches have shown high rates of disease-free survival [102].

3.4.2 Treatment of advanced, castration-resistant prostate cancer

While for clinically localized, early-stage disease the treatment is often curative, the same panorama is not evident for advanced, castration-resistant PCa [83].

Androgen deprivation therapy is the first-line treatment for patients with metastatic disease and is based in chemical castration (luteinizing hormone-releasing hormone drugs) or surgical castration (orchiectomy) [105]. However, the most part of the patients develop progressive disease after a median response to therapy of 18-30 months, which culminates in castration resistant PCa [106]. This stage is ultimately untreatable, even after therapy with the most effective chemotherapeutic agents, such as docetaxel, estramustine or prednisone [107, 108]. In addition, many patients develop metastases, usually bone metastases, responsible for excruciating pain that is not mitigated with chemotherapy [109]. Once spread, PCa is not curable and all therapy currently available is merely palliative.

3.5 Epigenetic alterations in prostate cancer

Although epigenetic mechanisms have a very important role in gene expression regulation in normal mammalian cells, in neoplasic cells, the normal patterns are altered. Indeed, the so called ‘epimutations’, alongside with genetic alterations, greatly contributes to malignant transformation.

Remarkably, PCa, as well as other tumor models, is the perfect paradigm of epigenetic catastrophe, since deregulation of a wide array of epigenetic mechanisms have been found in this disease, promoting cancer development and progression [5].

3.5.1 The DNA methylation profile in prostate cancer

Hypermethylation of DNA is one of most studied epigenetic deregulation in PCa, leading to gene silencing and loss of gene function, contributing to tumor initiation, progression and metastasis. *GSTP1* gene is probably the gene which promoter is more often reported as hypermethylated in PCa, leading to transcriptional silencing [110]. Being a very important enzyme in cellular metabolism, glutathione S-transferase P, encoded by *GSTP1*, is responsible for the elimination of reactive chemical species and protects the cell DNA from damage [111]. The loss of expression of *GSTP1* is found in PCa tissue and in tissues with PIN lesions, but not in normal prostate tissue or tissue from benign prostate hyperplasia [110, 112-114].

Another gene that is usually hypermethylated in PCa is the E-cadherin gene (*CDH1*) and loss of its transcriptional activity leads to transition from adenoma to carcinoma and acquisition of metastasic ability [115]. Actually, loss of E-cadherin expression have been linked to higher tumor grade [116]. As same as for *GSTP1*, *CDH1* is transcriptionally silenced due to hypermethylation of its promoter and treatment with demethylating agents, such as 5-aza-2'-deoxycytidine (DAC), have been reported to restore expression of E-cadherin levels [117].

Additionally, the androgen receptor (AR), activated by androgens, have been also reported to be regulated by DNA methylation in PCa. Although PCa is androgen dependent in its initial stages, it becomes androgen independent, due to gene promoter silencing. In AR-negative PCa cell lines, such as DU145, which promoter of *AR* gene is hypermethylated, in constrast with AR-positive PCa cell line LNCaP, gene re-expression has been achieved after treatment with DAC [118].

Furthermore, *CD44* is a gene which promoter is also hypermethylated in PCa. The glycoprotein codified by this gene is relevant for cell matrix adhesion, thus having an important role in cancer progression and metastasis [119]. Likewise, *APC* gene promoter was found to be hypermethylated in PCa, being a predictor of PCa progression and an important biomarker for prognosis evaluation [120]. Thus, currently more than 50 cancer-

related genes linked to important cellular pathways have been already found to be hypermethylated in PCa and therefore playing a critical role in neoplastic transformation [121].

3.5.2 Histone marks pattern

Focusing on global histone modification, there are patterns that predict the risk for PCa recurrence. PTMs in histone H3, such as H3K4me2 and H3K18ac, have been proposed as biological markers for PCa [122]. In addition, during tumor progression, high levels of epigenetic promoter silencing have been observed, resulting in permanent gene-silencing in neoplastic cells and their progeny [4]. This silencing of tumor suppressor genes has been associated with a particular combination of histone marks, such as: deacetylation of histones H3 and H4, loss of H3K4me, H3K9me2 and H3K9me3 and gain of H3K9me and H3K27me3 [123]. In fact, low levels of two of those marks – AcH3 and H3K9me – have been proposed to accurately distinguish between malignant and non-malignant samples. In addition, recently it was reported that high levels of H3K18Ac and H3K4me2 were indicative of a three-fold risk of cancer relapse/recurrence [124].

3.5.3 H2A.Z acetylation is fundamental for oncogenes expression in prostate cancer

As previously mentioned, H2A.Z has been associated with the carcinogenic process, mostly in breast cancer. Regarding PCa, it has been previously reported a significant increase of H2A.Z in castration-resistant LNCaP xenograft model of PCa. In fact is has been suggested that in a subset of primary prostate tumors the elevated expression of H2A.Z might be indicative of PCa progression to androgen independence [125].

Noticeable, a recent study evaluated how H2A.Z and its acetylated type interfere with transcription of oncogenes in PCa cell lines. In the same study the authors demonstrated that AcH2A.Z mutually excluded DNA methylation and H3K27me3, but was also tightly associated with active gene transcription. In fact, this posttranslational modification of H2A.Z tended to accumulate within the TSS of active genes. In contrast, in genes with lower activity during carcinogenesis, such as tumor suppressor genes, H2A.Z

deacetylation was mostly prevalent in nucleosomes next to the TSS and correlated with lower gene transcription activity [126].

Furthermore an activation/repression mechanism have been recently established (*Figure 12*).

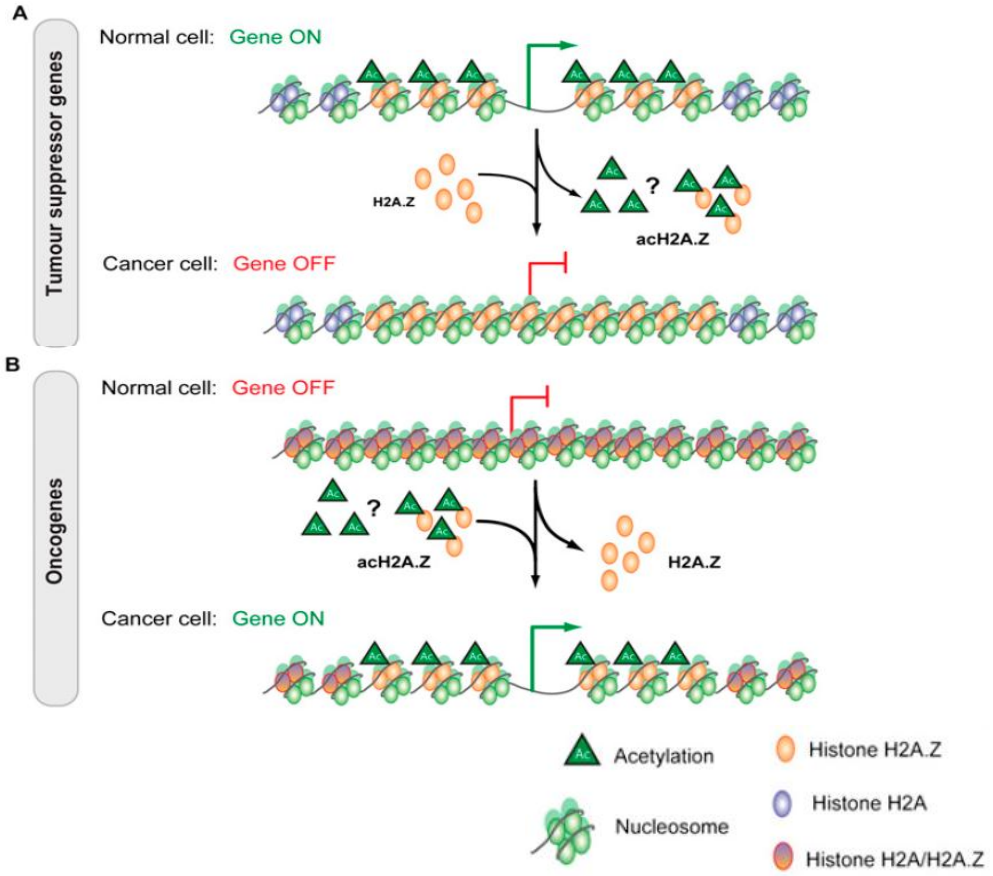


Figure 12 – Model of gene transcription regulation dependent of H2A.Z occupancy in PCa. **(A)** H2A.Z, by some uncertain mechanism, become hypoacetylated or is replaced by the deacetylated form of H2A.Z, leading to decreased expression of tumor suppressor gene. **(B)** Surrounding the TSS of oncogenes, after neoplastic transformation, H2A.Z become acetylated or it is substituted by its acetylated form, promoting the expression of this genes (Valdés-Mora *et al.*, 2012 [126]).

Nevertheless, in PCa the studies concerning H2A.Z are limited and solid information is not available yet.

3.6 Epigenetic modulating drugs and their importance in prostate cancer

One of the major features of epigenetic regulation is their reversible potential, a characteristic that is not shared by genetic alterations. Hence, this premise appears as an attractive for epigenetic treatment. Epigenetic modulating drugs have the capacity to

remodel the chromatin leading to re-expression of genes that might be silenced during tumor initiation and progression.

3.6.1 DAC and its relevance as DNA methyltransferase inhibitor

DAC exerts its antineoplasic activity by incorporating itself into newly synthetized DNA, during S-phase (*Figure 13*) [127]. At high doses its cytotoxic activity is due to covalent trapping off DNMT into DNA [128]. But, at lower doses, the mechanism is slightly different: the anti-oncogenic action results from its capacity to inhibit DNA hypermethylation and to reactivate tumor suppressor genes [127, 128]. DAC is phosphorylated and then incorporated into DNA, covalently binding to DNMT, acting as an irreversible inhibitor of the enzymatic activity of DNMTs [129].

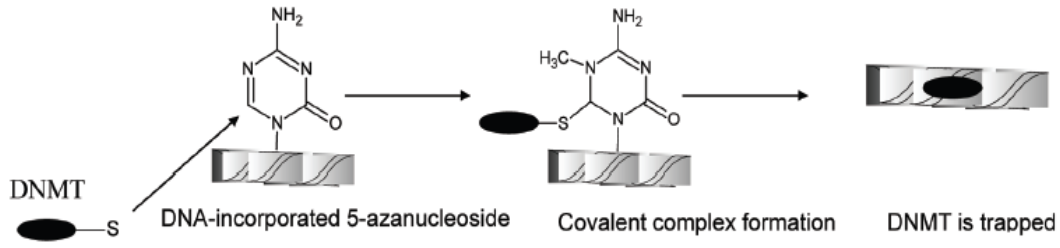


Figure 13 – Representation of the mechanism of action of 5-aza-2'-deoxycytidine, which is incorporated during DNA synthesis, replacing the standard cytidine (adapted from Fandy *et al.*, 2009 [130]).

It seems that there is a cascade of biochemical events initiated by promoter DNA methylation that involves initial DNA binding proteins, which attracts HDACs and HMTs, modifying histones into a silenced chromatin state, thus creating a silencing loop. Hence, DAC might interrupt this loop, since it induces hypomethylation and reverses the silence histone code at tumor suppressor genes loci [131, 132]. Actually, the effects of this drug on the histone code are not confined to genes showing silencing by promoter-associated methylation [133]. DAC also leads to enhanced acetylation of histones H3 e H4 at the promoter regions [134].

Although some studies regarding PCa therapy have been performed, it is important to mention that it is not well understood how and in which genes does DAC acts. Indeed, some experiments were performed to access the effect of this molecule in preventing gene

promoter methylation and it has been showed that DAC, induced re-expression of AR, estrogen receptor, progesterone receptor, E-cadehrin, CD44 and several genes of Y-chromosome, despite not preventing DNMT and BCL_2 expression[135-137].

3.6.2 Trichostatin A (TSA) and its potencial as HDACi

HDAC are complexes that enzymatically remove the acetyl group from lysine residues of the amino-terminal tails of histones, thus maintaining chromatin in a transcriptionally inactive state. This transcriptional blockade can be overcome by HDACi (*Figure 14*).

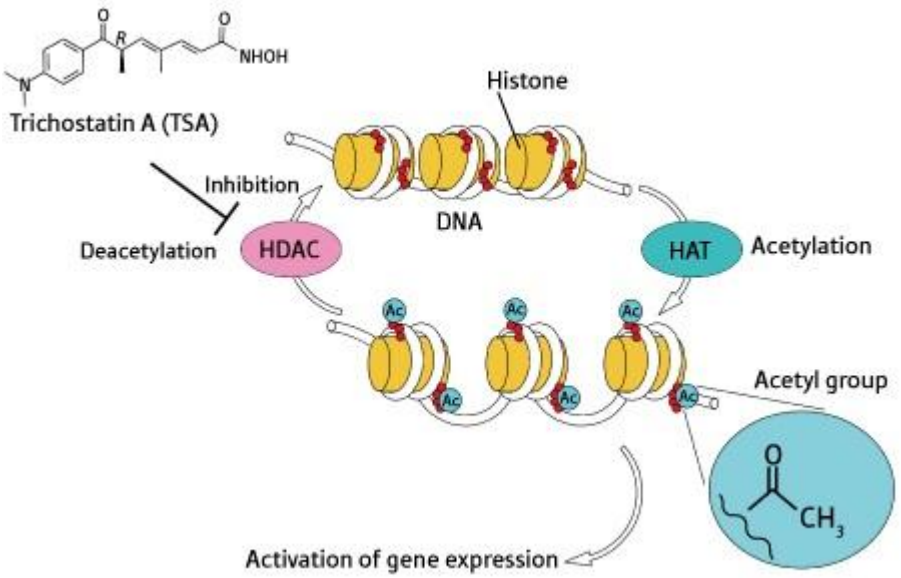


Figure 14 – TSA inhibits the activity of HDAC, allowing the histones to remain acetylated, bringing the chromatin into a transcriptional active state (adapted from Nasu, 2008 [138]).

A diversity of agents exhibit HDAC inhibitory activity and may have antitumor activity. Amongst the most usual HDACi used are TSA, sodium butyrate, depsipeptide, valproic acid, MS-275, SAHA, pyroxamide and phenylbutyrate. TSA was the first hydroxamic acid HDACi identified and it is a potent, but reversible, HDAC inhibitor, since it binds strongly to the HDAC catalytic site [139] (*Figure 15*).

TSA induces cell death and inhibits growth and proliferation of PCa cell line, since it inhibits the expression of *AR* [140, 141], and inhibits ERα-positive breast cancer cells' growth *in vitro* and *in vivo* [142]. TSA enhances acetylation as well as the stability of the ERα protein and p300 protein (proteins that may contribute to the treatment of human breast cancer) [142].

In vitro assays showed that TSA stabilized the site-specific acetylation of lysine 373 of p53 in LNCaP cells, which increased the basal levels of this proteins, a very well-known tumor suppressor protein, promoting cells' arrest at G_1/S, even though not inducing apoptosis [143, 144].

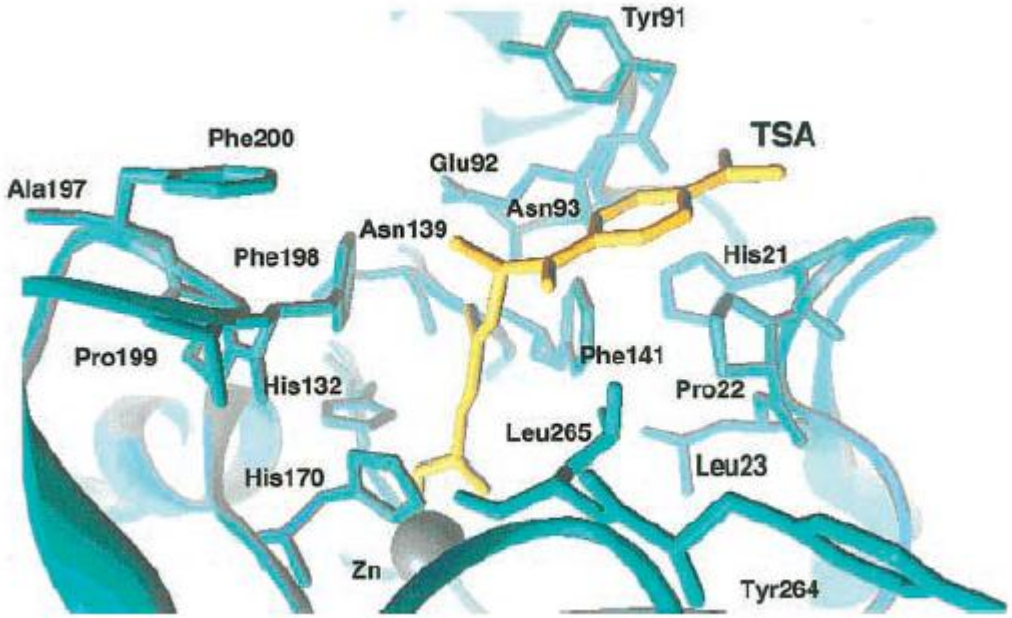


Figure 15 – Representation of how TSA interacts with the catalytic site of a HDAC homologue, forming a complex and inhibiting the catalytic capacity of the enzyme (Yoshida *et al.*, 2001 [145]).

Furthermore, *in vitro* experiments revealed that TSA induced hyperacetylation of X-ray repair cross-complementing protein 6 (Ku70) which decreases its ability to repair drug-induced DNA damage [146]. Interestingly, treatment of DU145 with both TSA and DAC has been found to be more effective in restoring functional expression of the *AR* and its downstream targets [146].

Currently, the implication of epigenetic deregulation in PCa is already acknowledged, either by site-specific DNA hypermethylation or histones' posttranslational modifications. However, information concerning the role of histone variants in carcinogenesis in PCa is rather poorly understood. Moreover, the effects of epigenetic treatment with HDACi and DNMTi in chromatin remodeling and histone variants need to be clarified. Therefore, there is a need to evaluate the impact of epigenetic modulating

drugs in non-canonical histones and nucleosome assembly regulation. In addition, a relation between sirtuin 1 and H2A.Z in cancer is yet to be reported, being this putative interaction an important mechanism of nucleosome assembly.

III
AIMS

III – AIMS

The effect of epigenetic drugs on the transcription of cancer-related genes is well known and its effectiveness might allow for the design of novel and alternative therapeutic approaches for cancer treatment. Since the carcinogenic role of H2A.Z has been previously documented in several tumor models, the main goal of this study was to evaluate the effect of epigenetic modulating drugs on the regulation of H2A.Z expression in prostate cancer.

The specific aims of this work were:

- ⇒ Evaluate mRNA and protein levels of *H2AFZ* in PCa cells lines after exposure to epigenetic modulating drugs;
- ⇒ Correlate the previous results with histone post-translational modifications of *H2AFZ* promoter;
- ⇒ Assess mRNA and protein levels of *SIRT1* in PCa cells lines after exposure to epigenetic modulating drugs and correlate them with histone post-translational modifications of *SIRT1* promoter;
- ⇒ Determine whether H2A.Z is regulated by sirtuin 1 in PCa cell lines, through:
    - o Modulation of H2A.Z levels after pharmacological inhibition of sirtuin 1;
    - o Assess H2A.Z levels after pharmacological activation of sirtuin 1;
- ⇒ Assess the role of epigenetic modulating drugs regarding its capacity to promote physical interaction between sirtuin 1 and H2A.Z;
- ⇒ Evaluate *H2AFZ* and *SIRT1* transcript levels in tissue samples and correlate the results with standard clinical and pathological parameters.

IV
MATERIAL AND METHODS

IV - Material and Methods

1. Cell Culture

Three PCa cell lines were selected for subsequent studies to evaluate the impact of epigenetic modulating drugs in *H2AFZ* and *SIRT1* transcript and protein levels. The chosen cell lines were LNCaP, DU145 and PC-3. PC-3 and LNCaP were acquire from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and DU145 cell line was obtained from American Type Culture Collection (ATCC, Lockville, MD, USA) and were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway.

All cell lines were grown in the recommended medium (*Table 3*), supplemented with 10% Fetal Bovine Serum (GIBCO®, Invitrogen, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin (GIBCO®, Invitrogen, Carlsbad, CA, USA). Cell lines were subcultered using Dissociation Reagent trypLE™ Express (GIBCO®, Invitrogen, Carlsbad, CA, USA) to harvest them as many times as necessary to obtain the desired number of 75cm^3 cell culture flasks.

Table 3 – Prostate cancer cell lines features and requirements for growth [147].

Cell Line	Origin	AR Status	Metastasic Potencial	Culture Medium
LNCaP	Derived from the left supraclavicular lymph node metastasis	Androgen sensible	Low	RPMI
DU145	Derived from brain metastasis	Hormono-refractory	Moderate	MEM
PC-3	Derived from bone metastasis	Hormono-refractory	High	RPMI + F12

1.1 Cell lines treatment with epigenetic-modulating drugs

The three cell lines were grown and treated with a pharmacological DNMTi, DAC (Sigma-Aldrich, Germany), and/or with a pan-inhibitor of HDACs, TSA (Sigma-Aldrich, Germany).

To prepare the stock solution of DAC at a concentration of 100mM, 10mg of the drug were dissolved in 438µL of 50% acetic acid and stored at -80ºC. Right before the use of this drug, the stock solution was diluted in 1x PBS to a final concentration of 10mM. To prepare the solution of 1mM TSA, which was the solution used during treatments, the 5mM TSA solution was diluted with 1x PBS. Finally, both solutions were stored at -20ºC during the treatment process.

The cell lines were grown until 20 to 30% of confluence was reached in 75cm^3 cell culture flasks. The, medium containing the corresponding drug(s) was added accordingly. Culture medium and drug(s) were renewed every day according to the schedule represented in *Table 4.*

Table 4 – Treatment timetable with the epigenetic modulating drugs DAC and/or TSA for three PCa cell lines (LNCaP, PC-3 and DU145).

	Day 1	**Day 2**	**Day 3**	**Day 4**
Mock	15mL medium	15mL medium	15mL medium	Harvest
1 µM DAC	15mL medium + 1.5 µL DAC (10mM)	15mL medium + 1.5 µL DAC (10mM)	15mL medium + 1.5 µL DAC (10mM)	Harvest
0.5 µM TSA	15mL medium	15mL medium	15mL medium + 7.5 µL TSA (1mM)	Harvest
1 µM DAC + 0.5 µM TSA	15mL medium + 1.5 µL DAC (10mM)	15mL medium + 1.5 µL DAC (10mM)	15mL medium + 1.5 µL DAC (10mM) + 7.5 µL TSA (1mM)	Harvest

The *Mock* cells were used as controls as they were submitted to the medium change procedure but were not exposed to any drugs. All the treatments were done in triplicate. On the last day of treatment, after 72h of exposure to the drug(s), cells were harvested by trypsinization and centrifuged. Pellets were washed in 1x PBS. After discarding 1x PBS, cells were stored separately for RNA and protein extraction at -80ºC.

1.2 Cell lines treatment with nicotinamide and resveratrol

NIC (Sigma-Aldrich, Germany) was obtained in the solid form and was prepared immediately before its use. Thus, 1.221g of NIC were dissolved in 10mL of 1x PBS, in order to obtain a solution with a final concentration of 1M. This solution was stored at -20ºC and the treatment procedure is described in *Table 5.*

Table 5 - Treatment schedule with NIC, DAC and TSA for three PCa cell lines (LNCaP, PC-3 and DU145).

	Day 1	**Day 2**	**Day 3**	**Day 4**
Mock	15mL medium	15mL medium	15mL medium	Harvest
20mM NIC	15mL medium + 300 µL NIC (1M)	15mL medium + 300 µL NIC (1M)	15mL medium + 300 µL NIC (1M)	Harvest
0.5 µM TSA + 20mM NIC	15mL medium + 300 µL NIC (1M)	15mL medium + 300 µL NIC (1M)	15mL medium + 7.5 µL TSA (1mM) + 300 µL NIC (1M)	Harvest
1 µM DAC + 0.5 µM TSA + 20mM NIC	15mL medium + 1.5 µL DAC (10mM) + 300 µL NIC (1M)	15mL medium + 1.5 µL DAC (10mM) + 300 µL NIC (1M)	15mL medium + 1.5 µL DAC (10mM) + 7.5 µL TSA (1mM) + 300 µL NIC (1M)	Harvest

RES (Sigma-Aldrich, Germany) was used since it has been described as an activator of sirtuin 1. The 100mM stock solution of RES was prepared dissolving 100mg in absolute ethanol and aliquots were stored at -20ºC. Right before its use, the 100mM RES solution was diluted with ethanol until a final concentration of 10mM, being this last solution the one used along the treatment procedure, as described in *Table 6*.

Table 6 – Treatment schedule with RES, DAC and TSA for three PCa cell lines (LNCaP, PC-3 and DU145).

	Day 1	**Day 2**	**Day 3**	**Day 4**
Mock	15mL medium	15mL medium	15mL medium	Harvest
40µM RES	15mL medium + 60µL RES (10mM)	15mL medium + 60µL RES (10mM)	15mL medium + 60µL RES (10mM)	Harvest
0.5 µM TSA + 40µM RES	15mL medium + 60µL RES (10mM)	15mL medium + 60µL RES (10mM)	15mL medium + 7.5 µL TSA (1mM) + 60µL RES (10mM)	Harvest
1 µM DAC + 0.5 µM TSA + 40µM RES	15mL medium + 1.5 µL DAC (10mM) + 60µL RES (10mM)	15mL medium + 1.5 µL DAC (10mM) + 60µL RES (10mM)	15mL medium + 1.5 µL DAC (10mM) + 7.5 µL TSA (1mM) + 60µL RES (10mM)	Harvest

In both cases as previously described the *Mock* cells were used as controls as they were submitted to the medium change procedure but were not exposed to any of the selected drugs. All the treatments were done in triplicate. On the last day of treatment, after 72h of exposure to the drug(s), cells were harvested by trypsinization and centrifuged.

Pellets were washed in 1x PBS. After discarding 1x PBS, cells were stored separately for RNA and protein extraction at -80ºC.

2. Expression analysis

2.1 RNA extraction and quantification

Total RNA extraction was performed using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. Briefly, cell pellets were thawed on ice, 1mL of TRIzol® Reagent was added to each sample and cells were homogenized using a syringe. The homogenized samples were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. After the incubation, all content of the tubes was transferred to 1.5mL RNAse-free tubes, 200µL of chloroform were added, the tubes vortexed for 15 seconds and incubated at room temperature for 3 minutes, followed by a centrifugation for 15 minutes at 10,600rpm, at 4ºC. Ended the centrifugation, it was possible to distinguish a lower red, phenol-chloroform phase, an interphase and an upper colorless aqueous phase enriched with RNA. Hence, the phase containing the RNA fraction was collected into a fresh RNAse-free tube and placed on ice. Afterwards, 500µL of isopropyl alcohol were added, the tubes vigorously inverted by hand and placed at room temperature for 10 minutes, to allow the RNA precipitation. Then, tubes were centrifuged for 10 minutes at 10,600rpm, at 4ºC, and the supernatant was discarded without disturbing the pellet. Finally, 1mL of 75% (v/v) ethanol was added to wash the RNA pellets by vortexing, followed by a centrifugation at 8,400rpm for 10 minutes, at 4ºC. The supernatant was carefully discarded and the RNA pellets were air-dried for 15-20 minutes. RNA pellets were eluted in a variable volume of RNA Storage Solution (1mM Sodium Citrate, pH 6.4) (Ambion, Applied Biosystems, Foster City, CA, USA) according to pellet size and placed on ice for at least 30 minutes before evaluation of RNA concentration and quality using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA was stored at -80ºC until further use.

2.2 DNAse treatment

In order to remove any genomic DNA present in our samples after RNA extraction, a treatment with DNase was performed, using TURBO DNA-free™ (Ambion, Applied Biosystems, Foster City, CA, USA) according to the protocol suggested by manufacturer. In short, 10µg of our previously extracted RNA were aliquoted into fresh-RNAse free 500µL tubes and DEPC-treated water (MP Biomedicals, OH, USA) was added until a final volume of 50µL was reached. Afterwards, 5µL of 10x TURBO DNase I Buffer and 1µL of TURBO DNase were added and an incubation of 30 minutes at 37ºC took place. Ended the incubation, 5µL of DNase Inactivation Reagent were added, tubes were vigorously mixed, incubated at room temperature for 2 minutes and centrifuged at 10,000 x g for 90 seconds. Finally, supernatant was carefully removed and stored into fresh, RNAse-free tubes.

2.3 cDNA synthesis

For gene expression analysis, cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's protocol. Hence, and briefly explained, in each RNAse-free PCR tube, which were packed on ice, were added 1000ng of template RNA (thawed on ice), 1x RT random primers, 1x RT buffer, 1x dNTP mix (4mM), 1µL of RNAse inhibitor, 1µL of Multiscribe™ Reverse Transcriptase and DEPC-treated water (MP Biomedicals, LLC, OH, USA) to complete a total volume of 20µL. All the components were gently mixed and the mixture was incubated at 25ºC for 10 minutes, followed by 37ºC for 120 minutes and, finally, 85ºC for 5 minutes. The incubation period was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Tubes were chilled on ice and 200µL of DEPC-treated water (MP Biomedicals, OH, USA) were used to dilute newly synthetized cDNA. All this procedure was also applied to Human Total RNA (Applied Biosystems, Foster City, CA, USA), that was used as a positive control for qRT-PCR assay further described. In this case, cDNA was diluted in 100µL of DEPC-treated water (MP Biomedicals, OH, USA). All samples were stored at -20ºC until further use.

2.4 Quantitative reverse transcriptase PCR (qRT-PCR)

H2AFZ and *SIRT1* transcripts were quantified by real time quantitative PCR. The assays were performed using gene expression assays for *H2AFZ* (Hs01888362_g1, Applied Biosystems, Foster City, CA, USA), *SIRT1* (Hs01009005_m1, Applied Biosystems, Foster City, CA, USA) and the endogenous control *GUSB* (Hs99999908_m1, Applied Biosystems, Foster City, CA, USA), being the last one used to normalize cDNA input. The expression assays were performed separately in 96-well plates in 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol.

In brief, in each well 9µL of previously synthesized cDNA, 1µL of TaqMan® Gene Expression Assay and 10µL of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were added. PCR conditions were those previously defined by manufacturer: 50ºC for 2 minutes, 95ºC for 10 minutes, 45 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. All cDNA samples were run in triplicate. cDNA synthesized from Human Total RNA (Applied Biosystems, Foster City, CA, USA) was used to prepare five consecutive cDNA dilutions (dilution factor of 10x) that were used as standards on each plate, allowing the construction of a standard curve for relative quantification and PCR efficiency assessment. Furthermore, multiple water blanks were added to each plate as negative controls.

The results were analysed using the 7500 Software for 7500 and 7500 Fast Real Time PCR Systems version 2.0.6 (Applied Biosystems, Foster City, CA, USA). A run was considered valid when the slope of the corresponding standard curve was above -3.60 (corresponding to a PCR efficiency >90%) and the R^2 of at least three relevant points exceeded 0.98.

For each sample, the mean quantity of *H2AFZ* and *SIRT1* expression levels were normalized against the mean quantity of *GUSB* expression levels for the corresponding sample. This ratio was then multiplied by 1000 for easier tabulation (Target Gene Expression Level = (Target Gene Mean Quantity / *GUSB* Mean Quantity) x 1000). Results were further presented as fold variation in comparison to our experimental control (*Mock*).

3. Protein levels analysis

3.1 Nucleus isolation

In order to inhibit protein degradation, all the procedure took place at 4ºC. Briefly, after harvesting, cells were twice washed in ice-cold 1x PBS, followed by a centrifugation at 1,500 x g for 5 minutes. Cell pellet was suspended in Buffer I (0.01M Tris-HCl, 0.01M NaCl, 0.003M MgCl2, 0.03M sucrose, pH 7 with the addition of 500µL of NP-40 for each 100mL of Buffer I prepared) and centrifuged at 1,500 x g for 10 minutes. Supernatant was promptly disposed and a new wash was performed in Buffer I, followed by a centrifugation. After the elimination of supernatant, two consecutive washes were carried out in 2mL of Buffer II (for each 100mL of Buffer I were added 1mL of 10mM $CaCl_2$) , each of them followed by a centrifugation at 1,500 x g. Finally, the nucleuses were purified and nuclear protein extraction was performed.

3.2 Nuclear protein extraction and quantification

Nuclear protein extraction from purified nucleus was performed using RIPA (Radio Immnuno Precipitation Assay) lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). An appropriated volume of complete RIPA Buffer (1x RIPA lysis Buffer, 1mM PMSF solution, 100mM sodium orthovanadate solution and 25x Protease Inhibitor Cocktail Solution) was added to the nucleus pellet, followed by vortexing to promote nuclear lysis. The lysates were transferred into a tube and incubated on ice for 15 min. After incubation, the tubes were centrifuged for 30 minutes at 13,000rpm at 4ºC to pellet the cell debris. The supernatant was collected to a new tube and the protein concentration was assessed by Qubit® 2.0 Fluorometer (Applied Biosystems, Foster City, CA, USA). Protein was stored at -80ºC until further use.

3.3 SDS-PAGE and Western Blot

Protein levels of H2A.Z and sirtuin 1 were evaluated by Western Blot using specific antibodies. The electrophoretic separation of proteins was performed on a polyacrylamide gel. The gel was prepared as described by Laemmli *et al.*: 12.5% running gel [12.5% (w/v) acrylamide/bis-acrylamide solution, 0.375M Tris-HCL pH8.8, 0,1%

(w/v) SDS, 0.1% (w/v) APS and 0.04% (v/v) TEMED] and 4% stacking gel [4% (w/v) acrylamide/bis-acrylamide solution, 0.062M Tris-HCL pH6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.25% (v/v) TEMED] [148]. Then, protein was suspended in loading buffer and denaturated at 95ºC for 5 minutes. The proteins were loaded in the running gel for separation and electrophoresis was performed in a drive Mini-Protean 3 Electrophoresis System (BioRad, Hercules, CA, USA), in running buffer (0.025M Tris, 0.192M glycine and 0.1% SDS, pH8.3) at 120V until the samples reached the bottom of the gel.

After their separation, proteins were blotted onto Immobilon®-P Transfer Membrane (Millipore, MA, USA). The system was mounted on a transfer unit Mini-PROTEAN 3 Electrophoresis System (BioRad, Hercules, CA, USA), according to manufacturer's instructions, and the transfer occurred at 50V at 4ºC for 3 hours in transfer buffer [0.025M Tris, 0.192M glycine, 20% (v/v) methanol]. After electroblotting, the membranes were incubated in blocking solution [5% (w/v) nonfat dry milk in 0.01M Tris-buffered saline containing 0.1% (v/v) Tween 20] for at least 1 hour, at room temperature with gentle shaking. The membranes were then incubated overnight at 4ºC in primary antibody – anti-H2A.Z (#2718, Cell Signaling Technology®, MA, USA) and anti-Sirtuin 1 (#1104-1, Epitomics®, CA, USA) – diluted 1:1000 in blocking buffer.

After the incubation with the primary antibody, the membrane was washed with Tween-TBS for 10 minutes with vigorous shaking, followed by two washes of 5 minutes each. Afterwards, membranes were incubated for 1 hour at room temperature with a Horseradish Peroxidase Conjugated Secondary Anti-Rabbit antibody (BioRad, Hercules, CA, USA). Then, membranes were washed with TBS-Tween as previously described. The membranes were developed using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad, Hercules, CA, USA) and exposed to Amersham Hyperfilm (GE Healthcare). To ascertain equal loading of protein, the membranes were stripped and reprobed with a monoclonal mouse antibody against β-Actin (Sigma- Aldrich®, Germany), diluted 1:8000 in blocking buffer.

Finally, films were digitalized and optical densities of visible bands were determined using QuantityOne® Software version 4.6.6 (Biorad, Hercules, CA, USA). Results were further presented as fold variation in comparison to our experimental control (*Mock*).

4. Chromatin immunoprecipitation (ChIP) and analysis by qRT-PCR

ChIP was performed using EZ-Magna ChIP™ G – One-Day Chromatin Immunoprecipitation Kit (Millipore, MA, USA) according to manufacturer's instructions.

4.1 In vivo crosslinking and lysis

LNCaP and DU145 cell lines were treated with DAC and TSA as previously mentioned. To each flask formaldehyde was added until a final concentration of 1% was reached, followed by a gentle swirl to mix the formaldehyde with culture medium. Afterwards, the flaks were left to incubate for 10 minutes at room temperature. 2mL of 10x glycine were added in each dish in order to quench unreacted formaldehyde, followed by a gentle swirl and an incubation of 5 minutes at room temperature. After the incubation time, flasks were placed on ice.

The culture medium was carefully removed and 20mL of cold 1x PBS were added in order to wash the cells. PBS was removed and a new wash was similarly performed. Finally, cells were scrapped and resuspended into microfuge tubes containing 2mL of cold 1x PBS and 1x Protease Inhibitor Cocktail II (PIC), which were then centrifuged at 800 x g for 5 minutes, at 4ºC, to pellet the cells. Supernatant was discarded and cells were suspended in 0.5mL of 1x Cell Lysis Buffer and 1x PIC, followed by an incubation on ice for 15 minutes, making sure that tubes were vortexed every 5 minutes. Tubes were centrifuged again (800 x g for 5 minutes, at 4ºC), supernatant was removed and 0.5mL of 1x Nuclear Lysis Buffer and 1x PIC were added and the pellet resuspended.

4.2 Sonication to shear DNA

For optimal sonication, approximately 300µL of resuspended pellet were distributed into fresh microfuge tubes. Sonication was performed for 20 minutes on iced water using a Bioruptor® Standard (Diagenode, Philadelphia, PA, USA), with cycles of 20 seconds with sonication ON, followed by 50 seconds with sonication OFF. Sonicated chromatin was stored into fresh microfuge tubes in 50µL aliquots, at -80ºC, until further use.

In order to test the sonication, 5µL of sonicated chromatin was incubated with 10µg of RNase (Sigma-Aldrich, Germany) at 37ºC for 30 minutes, followed by an addition of 1µL of Proteinase K and an incubation at 62ºC for 2 hours. After that, sample was loaded

into a 2% agarose gel and run for 1 hour, at 140 V, and was observed in an ultraviolet transilluminator [Pharmacia Biotech ImageMaster VDS (Pharmacia Biotech, Bay Area)] in order to validate the sonication.

4.3 Immunoprecipitation of crosslinked protein/DNA

For a single immunoprecipitation it is required 450µL 1x Dilution Buffer and 2.25µL 1x PIC. In order to guarantee identical conditions between each immunoprecipitation, all sonicated chromatin was primarily treated into the same 15mL falcon tube. Hence, the 50µL aliquots of sonicated chromatin were diluted in the respective volume of Dilution Buffer with PIC. 5µL of this solution was reserved into a fresh tube to use as input control, being stored at 4ºC until elution of protein/DNA complexes and reverse crosslink of protein/DNA complexes to free DNA, as described below.

Afterwards, 450 µL of the previously made solution were deposited into fresh microfuge tubes and the antibodies were added [50µL of anti-H2A (ab18262, Abcam, Cambridge, UK), 5µL of anti-H2A.Z (ab4174, Abcam, Cambridge, UK), 10µL of anti-AcH2A.Z (ab18262, Abcam, Cambridge, UK), 5µL of anti-H3 (ab1791, Abcam, Cambridge, UK), 5µL of anti-AcH3 (06-599, Millipore, MA, USA), 5µL of anti-H3K4me2 (ab32356, Abcam, Cambridge, UK), 5µL of anti-H3K27me3 (07-499, Millipore, MA, USA)], 1µL of the positive control anti-RNA Polymerase II and 1µL of the negative control Normal Mouse IgG, the last two provided with the kit. Furthermore, 20µL of fully suspended protein G magnetic beads were added to the tubes. Microfuge tubes were then incubated overnight with rotation, at 4ºC.

On the next day, protein G magnetic beads were pelleted using a magnetic separator (Magma Grip Rack) (Millipore, MA, USA) and the supernatant was removed. After that, beads were fully resuspended and washed for 5 minutes, with constant rotation, with four different buffers in the following order:

- Low Salt immune Complex Wash Buffer;
- High Salt Immune Complex Wash Buffer;
- LiCl Immune Complex Wash Buffer;
- and TE Buffer.

4.4 Elution of protein/DNA complexes and reverse crosslink of protein/DNA complexes to free DNA

In each tube were added 100µL of ChIP Elution Buffer and 1µL of Proteinase K and they were incubated for 2 hours at 62ºC, with permanent shaking. Afterwards, tubes were incubated during 10 minutes, at 95ºC, and they were allowed to cool until they reached room temperature. Beads were separated using the magnetic separator and the supernatant was reserved into fresh microfuge tubes.

4.5 DNA purification using spin columns

For each immunoprecipitation, 500µL of Bind Reagent "A" were added to a Spin Filter within a Collection Tube. Tubes were centrifuged at 12,600 x g for 30 seconds, and the eluate was discarded (both filter and collection tube were saved). 500µL of Wash Reagent "B" were added into the same Spin Filter, a new centrifugation was performed (12,600 x g for 30 seconds), the eluate was again discarded and another centrifugation took place (12,000 x g for 1 minute). Finally, the Spin Filter was put into a fresh collection tube, 50 µL of Elution Buffer "C" were added directly onto the center of the white Spin Filter Membrane and a centrifugation was performed (12,600 x g for 30 seconds). The Spin Filter was discarded and the Collection Tube containing the purified DNA was stored at -20ºC until further use.

4.6 Real-time quantitative PCR (qRT-PCR)

A real-time quantitative PCR was performed in order to analyze the specific prost-translational histone marks nearby *H2AFZ* and *SIRT1* gene promoters. Therefore three pairs of primers were designed for each gene, being the primers "A" those closer to the TSS and primers "C" those which were farthest from the TSS. Information about the primers used is compelled in *Table 5*.

Table 7 – qRT-PCR primers features: sequence, distance from TSS and annealing temperature.

Gene	Primer		Sequence	Distance from TSS	$T_{ANNEALING}$ (ºC)
H2AFZ	A	Forward	5' agcgtagctcgctctgtttc 3'	228bp	60
		Reverse	5' atgagcaagcgaagaaaagg 3'		
	B	Forward	5' gtgacttggcttcaccgtt 3'	733bp	60
		Reverse	5' ttcttccgtctaggcgagtc 3'		
	C	Forward	5' agacactactgtccccaagca 3'	1140bp	60
		Reverse	5' gtccatgcaatgcaatcagt 3'		
SIRT1	A	Forward	5' tacacttcaggaagacgtggaa 3'	310bp	62
		Reverse	5' ccgctttctcaacttctctttc 3'		
	B	Forward	5' tgcacgtgagaaaactgagg 3'	714bp	62
		Reverse	5' acctttgacgtggaggtttg 3'		
	C	Forward	5' tatggccagaacccatactagg 3'	1127bp	60
		Reverse	5' ggacccatataacccatggtag 3'		

The assays were performed separately in 96-well plates in 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol.

In short, in each well 2µL of sonicated chromatin, 10µL of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1µL of primers solution (forward + reverse) (10mM) and 7µL DEPC-treated water (MP Biomedicals, OH, USA) were added. PCR conditions were those previously defined by manufacturer: 50ºC for 2 minutes, 95ºC for 10 minutes, 45 cycles at 95ºC for 15 seconds and 60/62ºC for 1 minute, 95ºC for 15 seconds, 60/62ºC for 20 seconds and, finally, 95ºC for 15 seconds.

All samples were run in triplicate. gDNA extracted from LNCaP cell line was used to prepare five consecutive gDNA dilutions (dilution factor of 10x) that were used as standards on each plate, allowing the construction of a standard curve for relative quantification and PCR efficiency assessment. Furthermore, multiple water blanks were added to each plate as negative controls.

The results were analyzed using the 7500 Software for 7500 and 7500 Fast Real Time PCR Systems V2.0.6 (Applied Biosystems, Foster City, CA, USA). A run was considered valid when the slope of the corresponding standard curve was above -3.60 (corresponding to a PCR efficiency >90%) and the R^2 of at least three relevant points exceeded 0.98.

After the experiment, ChIP-qPCR needs to be normalized. Normalization was conducted using the *Input Percent Method*, where signals obtained from the ChIP were divided by signals obtained from the input sample, the last one representing the amount of chromatin used for immunoprecipitation.

5. Proximity ligation assay (PLA)

One PCa cell line, PC-3, cultured in 1cm^2 coverslips, was exposed to TSA, alone or combined with DAC, and to RES, as previously mentioned. Additionally, *Mock* cells were used as controls, since they were submitted to the medium change procedure, but were not exposed to any of the selected drugs.

Ended the treatment procedure, cells were fixed in 4% formaldehyde (Sigma-Aldrich, Germany) for 10 minutes, and subsequently stored in methanol, at -20ºC, until further use. Immediately before PLA procedure, cells were permeabilized in 0.5% Triton X-100 (Sigma-Aldrich, Germany), for 5 minutes, at room temperature and with gentle mix.

PLA assay was performed using the commercial kit Duolink® In Situ (OLINK Bioscience, Uppsala, Sweden), according to manufacturer's instructions. The antibodies used were anti-sirtuin 1 (ab110304, Abcam, Cambridge, UK) and anti-H2A.Z (#2718, Cell Signaling Technology®, MA, USA), diluted 1:200 and 1:50, respectively. After the procedure, slides were analysed using fluorescent microscopy (Olympus IX51, Olympus, UK).

6. Expression analysis in clinical samples

6.1 Sample collection

Fifty prostate tumor samples were prospectively collected from patients diagnosed for prostate carcinoma. Those patients were diagnosed and treated with radical prostatectomy at the Portuguese Oncology Institute – Porto, Portugal. The ten morphological normal prostate tissues, used as controls, were obtained from patients submitted to radical cystoprostatectomy for bladder cancer.

After surgery, all tissues specimens were promptly frozen at -80ºC and subsequently cut in a cryostat for nucleic acid extraction. The bulk material was routinely

fixed in buffered formalin and paraffin-embedded. The corresponding hematoxylin-eosin stained sections were examined by a pathologist to determine tumor type, grade classification, Gleason score and pathological stage according to the TNM staging system.

Relevant clinical data was collected from patient's clinical records. Patients for this study were enrolled after informed consent. These studies were approved by the institutional review board (Comissão de Ética) of Portuguese Oncology Institute – Porto, Portugal.

6.2 RNA Extraction

Total RNA extraction was performed in 50 prostate carcinoma samples and 10 normal prostatic tissue using PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 750µL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) were added to each 1.5mL tube containing tissue samples. Then, tissue was homogenized using a rotor-stator and 750µL of TRIzol® Reagent were added again. Tubes were incubated at room temperature for 8 minutes and, subsequently, 300µL of chloroform (Merck, Germany) were added. Tubes were hand shaken for 15 seconds and the mixture was incubated for 3 minutes at room temperature, followed by a 15 minutes centrifugation at 12,000 x g and 4ºC. Afterwards, 600µL of the upper, aqueous phase containing the RNA were transferred to a new RNAse-free tube, 600µL of 70% (v/v) of ethanol were added and tubes were vigorously shaken. Subsequently, 700µL of the previous solution were transferred to a Spin Cartridge, which was placed in a Collection Tube, and a centrifugation took place (12,000 x g for 15 seconds, at room temperature). The eluate was discarded and the remaining volume of the solution containing the aqueous phase and 70% (v/v) ethanol was transferred to the same Spin Cartridge and a new centrifugation was performed. When all the solution had been processed, 700µL of Wash Buffer I were added to the Spin Cartridge and it was centrifuged at 12,000 x g for 15 seconds, at room temperature. Then, the Spin Cartridge was placed onto a new Collection Tube and 500µL of Wash Buffer II were added. After centrifugation (12,000 x g for 15 seconds), another wash with Wash Buffer II was done, followed by a 1-minute centrifugation. Finally, the Spin Cartridge was placed in a new tube in order to recover the RNA by elution, and 50µL of RNAse-free water was added to the center of the membrane, followed by incubation for 3 minutes at room temperature and

a centrifugation for 2 minutes (12,000 x g, at room temperature). A new elution step was performed for a final volume of 100µL. RNA concentration and purity ratios were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA quality was assessed by a run in an agarose gel and RNA samples were stored at -80ºC until further use.

6.3 DNAse treatment

In order to remove any genomic DNA present in our samples after RNA extraction, treatment with DNase was performed, using *TURBO DNA-free*™ (Ambion, Applied Biosystems, Foster City, CA, USA) according to the protocol suggested by manufacturer, as previously described.

6.4 cDNA synthesis

For gene expression analysis, cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's protocol, as previously described.

6.5 Quantitative reverse transcriptase PCR (qRT-PCR)

H2AFZ and *SIRT1* transcripts were quantified by real time quantitative PCR. The assays were performed using pre-made gene expression assays for *H2AFZ* (Hs01888362_g1, Applied Biosystems, Foster City, CA, USA) and *SIRT1* (Hs01009005_m1, Applied Biosystems, Foster City, CA, USA) and the endogenous control *GUSB* (Hs99999908_m1, Applied Biosystems, Foster City, CA, USA), the latter used to normalize cDNA input. The expression assays were performed separately in 96-well plates in 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the recommended protocol, as previously described.

7. Statistical Analysis

In cell lines, differences in transcript levels of *H2AFZ* and *SIRT1* between the treatments performed were determined using an One-Way Analysis of Variance (one-Way

ANOVA) test, followed by a multiple comparison Dunnett's test, comparing all groups against the Mock. Differences regarding H2A.Z and sirtuin 1 protein levels were also evaluated using an One-Way Analysis of Variance (one-Way ANOVA) test, followed by a multiple comparison Dunnett's test, comparing all groups against the experimental control.

Differences in quantitative expression levels of *SIRT1* and *H2AFZ* between PCa samples and normal prostate tissues were assessed by the nonparametric Mann-Whitney U-test. The relationship between expression levels and other standard clinicopathological variables such as Gleason score and pathological stage were assessed using the Kruskal-Wallis or the Mann-Whitney tests, as appropriate.

All tests were two-sided and *p*-values were considered significant when inferior to 0.05. Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, CA, USA). Graphs were built using GraphPad Prism version 5.0 for Windows (GraphPad Software, CA, USA).

V
RESULTS

V - RESULTS

1. Epigenetic modulating drugs lead to an increase in H2AFZ transcript levels with a concomitant decrease of its protein levels

In order to investigate whether DAC and/or TSA might induce alterations in *H2AFZ* transcript levels, three PCa cell lines (LNCaP, PC-3 and DU145) were exposed to these drugs, either alone or in combination (*Figure 16*).

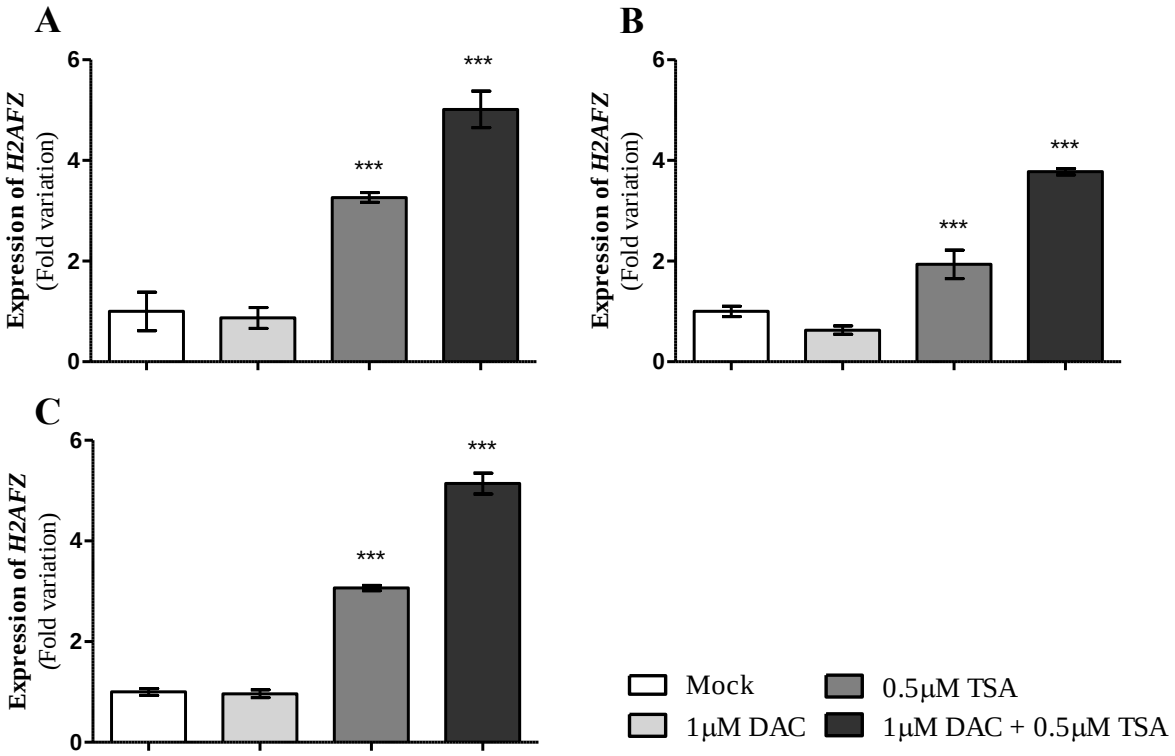


Figure 16 – Transcript levels of *H2AFZ* in three distinct cell lines (**A**- LNCaP, **B**- PC-3 and **C**- DU145) after exposure to DAC and/or TSA. The results are presented as fold variation in comparison to the experimental control.

Dunnett's test, *** $p<0.001$

DAC alone did not induce any alteration in *H2AFZ* transcript levels, whereas exposure to TSA alone or combined with DAC significantly increased the *H2AFZ* mRNA

levels in all tested cell lines (p<0.001). Indeed, the highest *H2AFZ* transcript levels were obtained after combined treatment.

Furthermore, protein levels of this histone variant were also assessed by Western Blot (*Figure 17*).

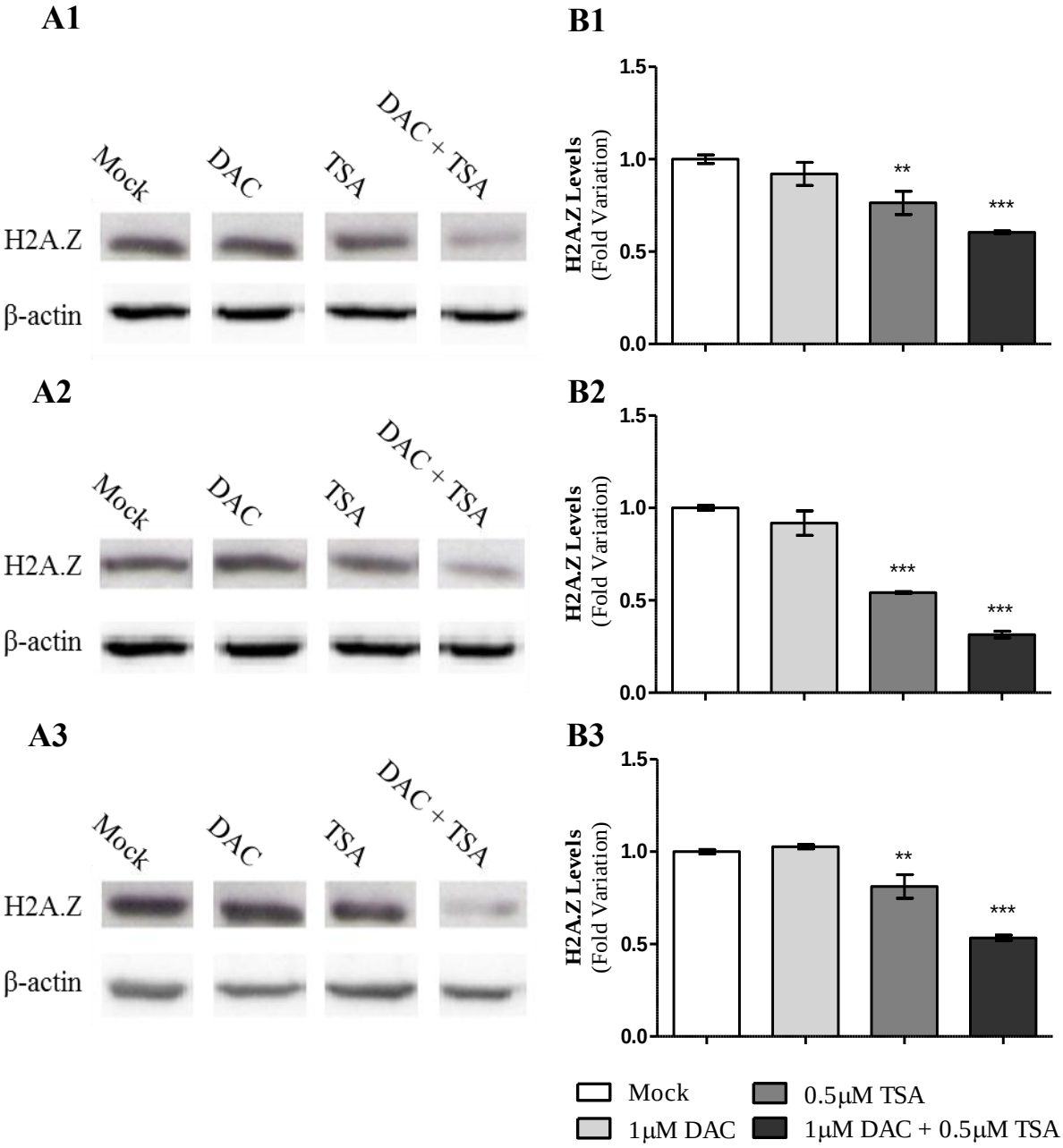


Figure 17 – Results for **(A)** Western Blot and **(B)** fold variation of optical density for H2A.Z protein levels in **(1)** LNCaP, **(2)** PC-3 and **(3)** DU145 cell lines. Results were normalized using the constitutive protein β-actin and presented as fold variation in comparison with *Mock*.

Dunnett's test, ** p<0.01; *** p<0.001

Unexpectadly, *H2AFZ* protein levels followed the opposite trend of the transcript levels as they significantly decreased after exposure to TSA ($p<0.01$ for LNCaP and DU145 and $p<0.001$ for PC-3) and TSA combined with DAC ($p<0.001$).

2. Cell lines exposure to TSA alone or combined with DAC induces enrichment in transcription activation marks of histones in the vicinity of H2AFZ transcription start site

After treatment with TSA alone or combined with DAC, chromatin immunoprecipitation was performed in LNCaP and DU145 cell lines. Since DAC alone did not affect *H2AFZ* transcript and protein levels, the effect of this treatment on histone marks was not further studied. Chromatin of treated cell lines was immunoprecipitated for several transcriptional activating histone marks (acH2A.Z, acH3 and H3K4me2) and for a repression histone mark (H3K27me3). Immunoprecipitation for H2A, H2A.Z and H3 was also performed. After ChIP, qRT-PCR was carried out to evaluate accumulation of marks along the *H2AFZ* promoter region (*Figures 18* and *19*).

Interestingly, for both LNCaP and DU145 cell lines, no relevant differences in H2A and H2A.Z levels were observed after treatment. However, H2A tended to accumulate in zones distant from the TSS while H2A.Z was enriched near the TSS. Remarkably, acH2A.Z increased mostly nearer to the TSS in PCa cells exposed to TSA. Contrarily, H3 levels did not change significantly along *H2AFZ* promoter in both cell lines after treatment. Concerning H3 activation marks, AcH3 and H3K4me2, an increase nearer to the TSS was observed after drug(s) exposure. Nevertheless, no variations were observed for the H3K27me3 repressive mark along the entire *H2AFZ* promoter.

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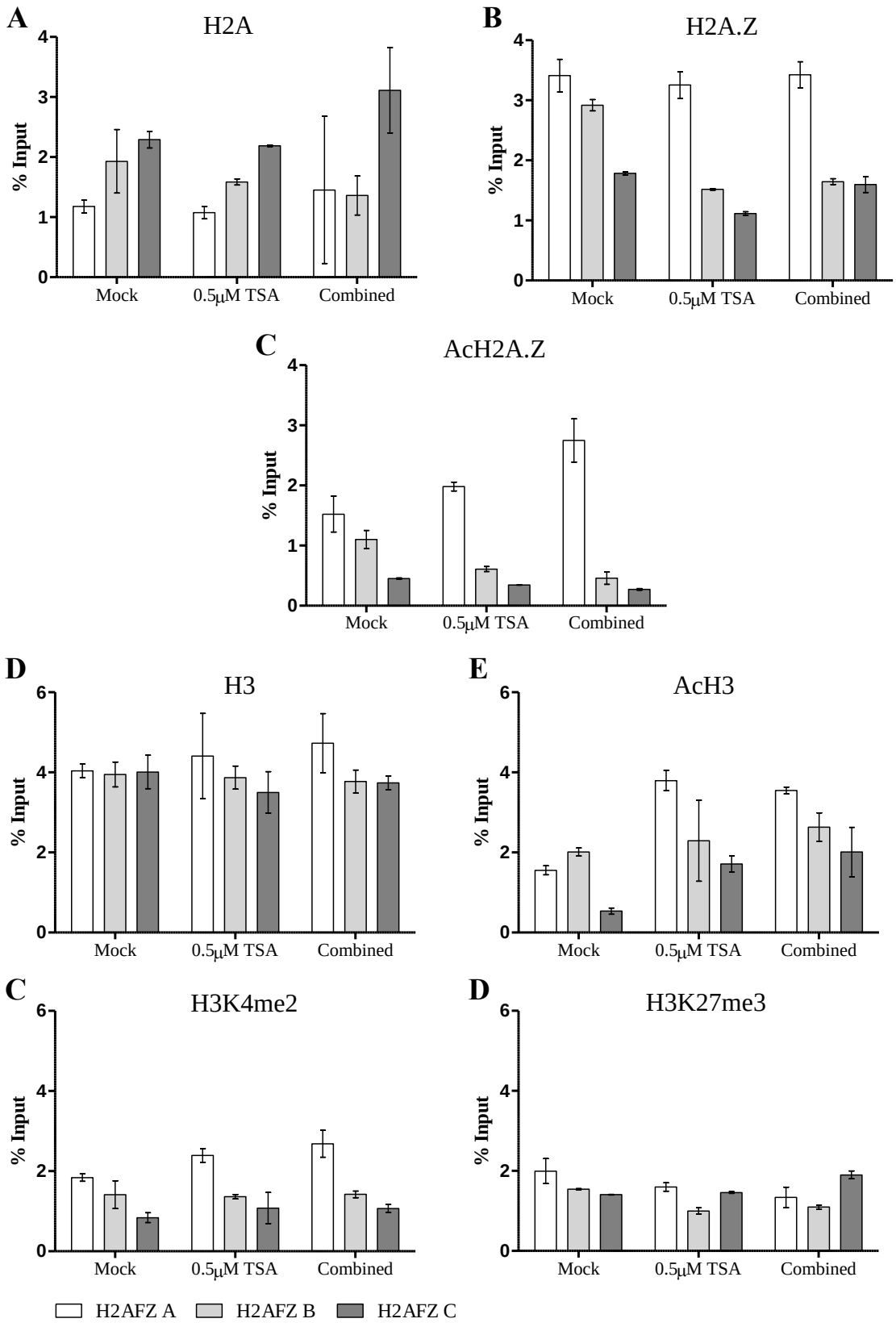

Figure 18 – ChIP assay results for LNCaP cell line regarding **(A)** H2A, **(B)** H2A.Z, **(C)** AcH2A.Z, **(D)** H3, **(E)** AcH3, **(F)** H3K4me2 and **(G)** H3K27me3 histones and histones marks across *H2AFZ* promoter. Results are normalized with the input of total sonicated chromatin

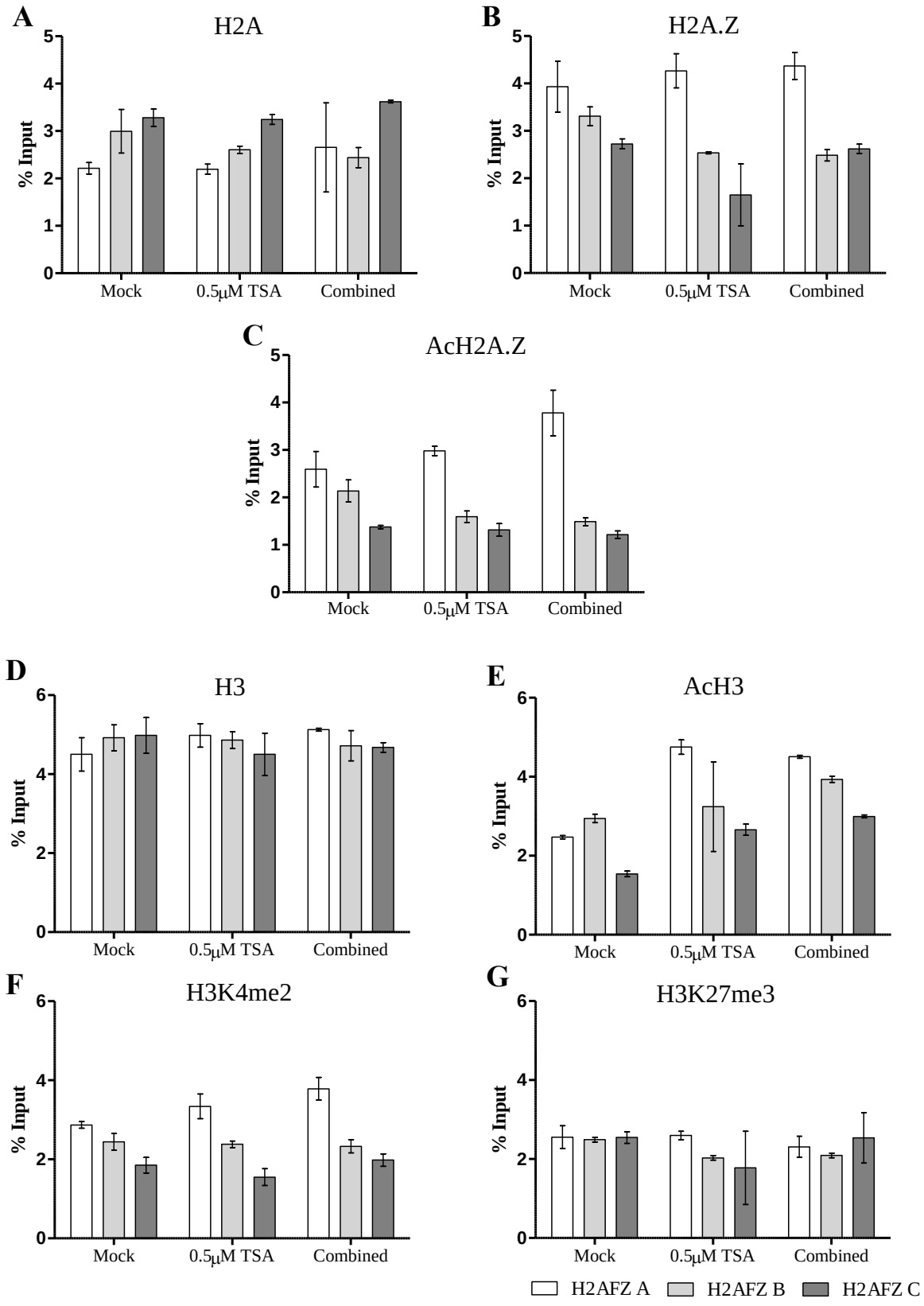

Figure 19 - ChIP results for DU145 cell line concerning **(A)** H2A, **(B)** H2A.Z, **(C)** AcH2A.Z, **(D)** H3, **(E)** AcH3, **(F)** H3K4me2 and **(G)** H4K27me3 histones and histones marks across *H2AFZ* promoter. Results are normalized with the input of total sonicated chromatin.

3. Epigenetic modulating drugs increase SIRT1 transcript and protein levels

Because it has been recently suggested that sirtuin 1 might degrade H2A.Z, transcript levels of *SIRT1* were also assessed in the same three cell lines which were exposed to epigenetic-modulating drugs (*Figure 20*).

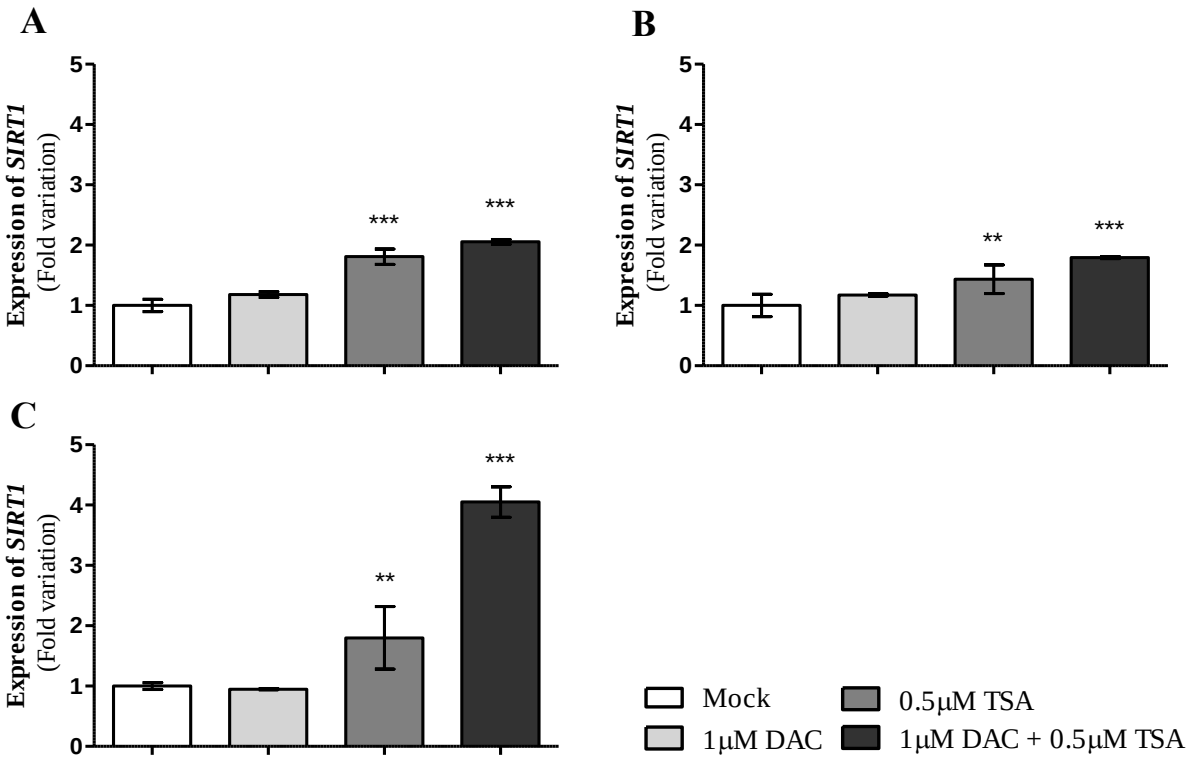

Figure 20 – Transcript levels of *SIRT1* in three distinct cell lines – **(A)** LNCaP, **(B)** PC-3 and **(C)** DU145 – after exposure to DAC and/or TSA. The results are presented as fold variation in comparison to our control.

Dunnett's test, ** $p<0.01$; *** $p<0.001$

Remarkably, whereas DAC alone did not alter *SIRT1* mRNA levels, a statistically significant increase was observed for all PCa cell lines exposed to TSA ($p<0.001$ for LNCaP and $p<0.01$ for PC-3 and DU124) and TSA combined with DAC ($p<0.001$ for all three cell lines).

Simultaneously, protein levels of sirtuin 1 were also assessed (*Figure 21*) and these significantly increased after TSA exposure in LNCaP and DU145 cell lines ($p<0.001$) and, more impressively, after exposure to DAC and TSA ($p<0.001$).

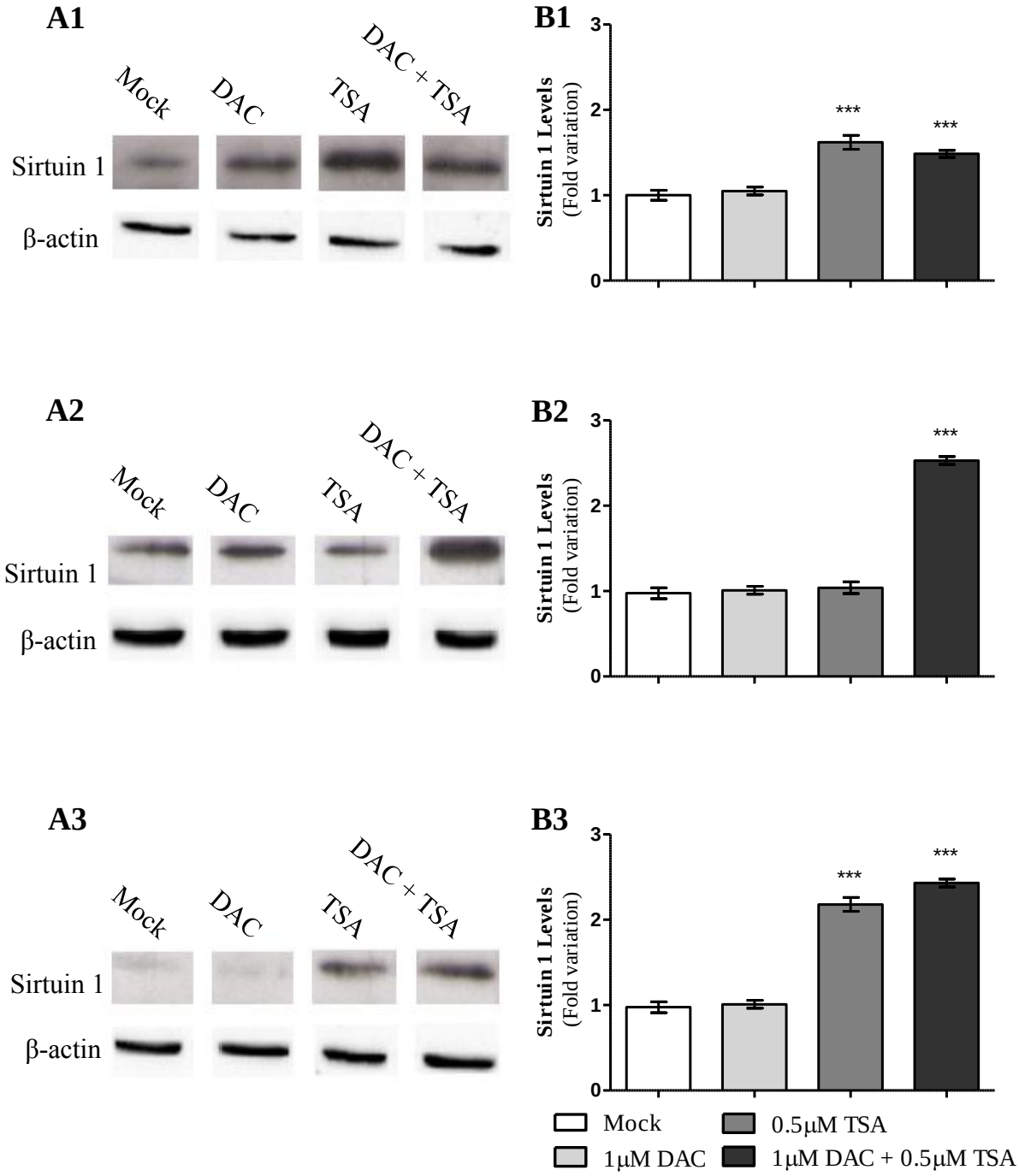

Figure 21 – Results for **(A)** Western Blot and **(B)** fold variation of optical density for H2A.Z protein levels in **(1)** LNCaP, **(2)** PC-3 and **(3)** DU145 cell lines. Results were normalized using the constitutive protein β-actin and presented as fold variation in comparison with the control.

Dunnett's test, *** $p<0.001$

4. Exposure of PCa cell lines to TSA alone or combined with DAC results in an enrichment of transcriptional activation marks in histones neighboring the SIRT1 transcription start site

Similar to the observations on the *H2AFZ* gene promoter, drug exposure did not alter the deposition of H2A and H2A.Z across the *SIRT1* gene promoter. However, H2A.Z was preferentially located at nucleosomes near the TSS, whereas H2A was preferentially located within the nucleosomes more distant from the TSS. Nonetheless, levels of AcH2A.Z were not altered by epigenetic treatment, and these were dispersed along the *SIRT1* promoter *(Figures 22 and 23).*

While in DU145 cell line the levels of H3 were stable along the promoter and after treatment, in LNCaP cells that trend was not so obvious, although relevant differences were not apparent. In addition, in both PCa cell lines there was an increase in marks responsible for gene transcription activation (AcH3 and H3K4me2) after drug(s) exposure, being more enriched in nucleosomes nearer to the TSS. Remarkably, the repressive mark H3K27me3 decreased after TSA exposure, either alone or in combination with DAC.

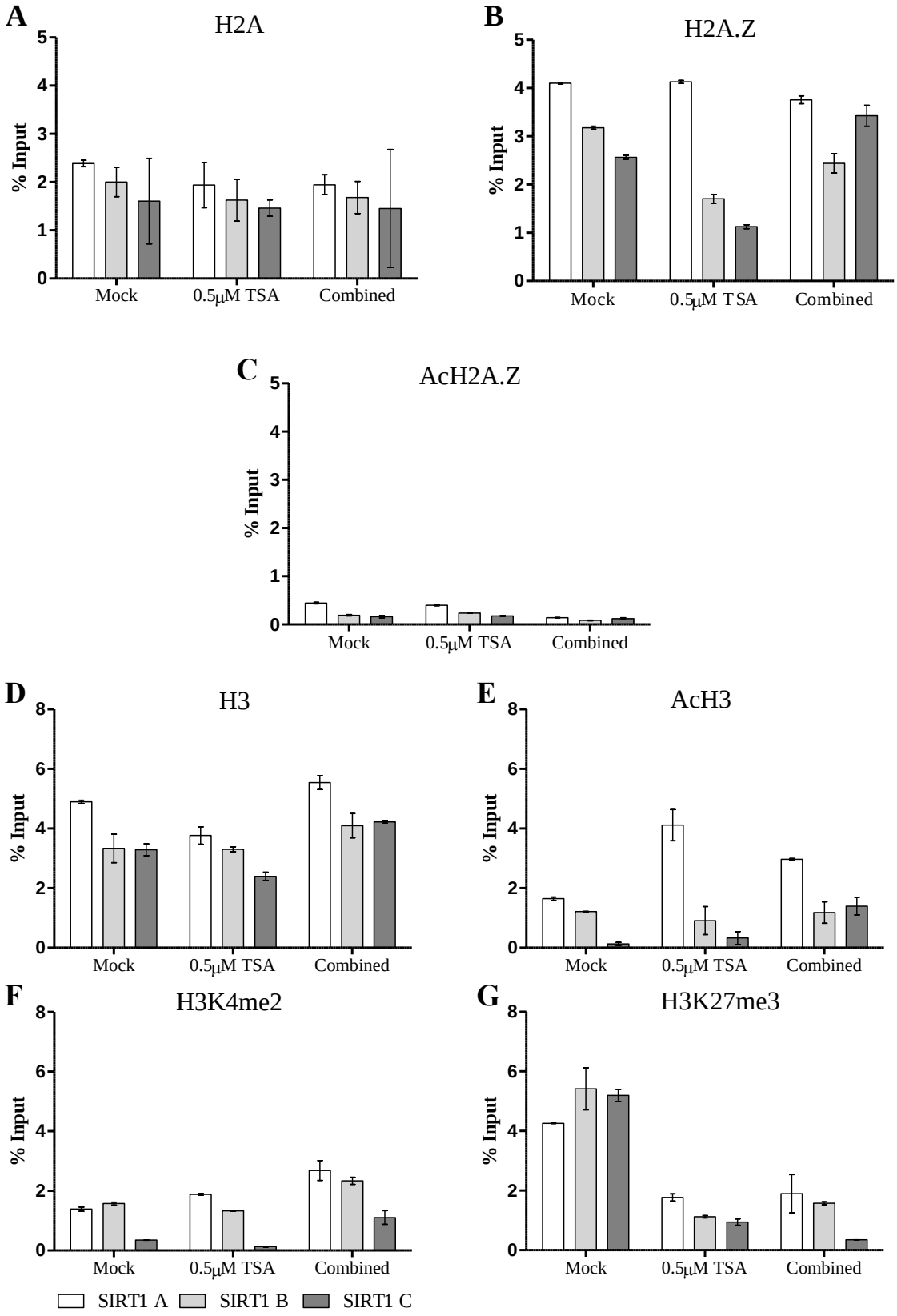

Figure 22 – ChIP for LNCaP cell line regarding **(A)** H2A, **(B)** H2A.Z, **(C)** AcH2A.Z, **(D)** H3, **(E)** AcH3, **(F)** H3K4me2 and **(G)** H4K27me3 histones and histone marks along *SIRT1* promoter. Results were normalized with the input of total sonicated chromatin.

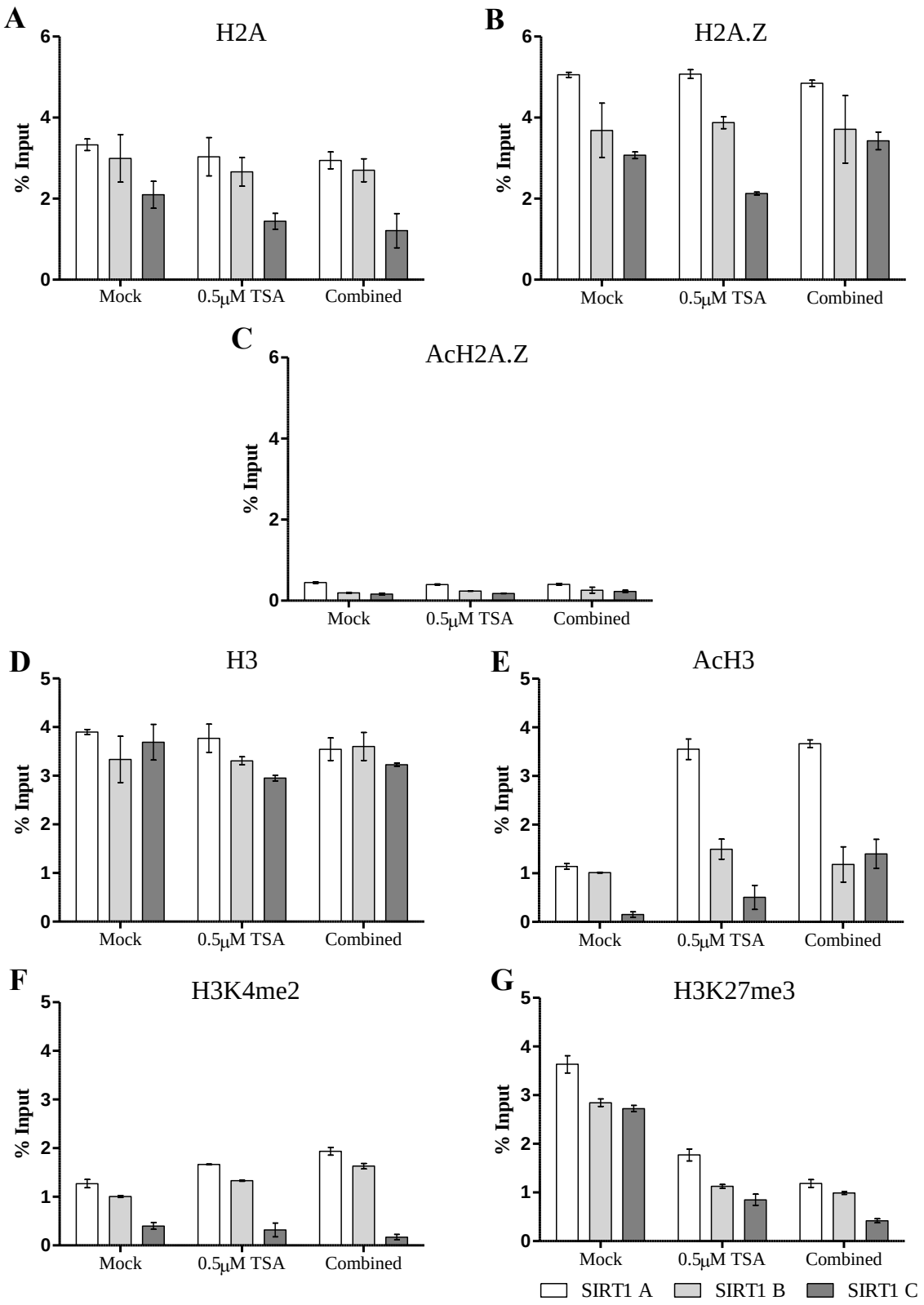

Figure 23 – ChIP results for DU145 cell line regarding **(A)** H2A, **(B)** H2A.Z, **(C)** AcH2A.Z, **(D)** H3, **(E)** AcH3, **(F)** H3K4me2 and **(G)** H4K27me3 histones and histones marks across *SIRT1* promoter. Results are normalized with the input of total sonicated chromatin.

5. Nicotinamide (NIC) exposure is associated with H2A.Z overexpression

To evaluate the effect of sirtuin 1 inhibition on H2A.Z protein levels, cell lines (LNCaP, PC-3 and DU145) were treated with the former epigenetic modulating drugs (TSA and DAC) and with NIC, a sirtuin 1 inhibitor. Western blot analysis was performed to determine protein levels profile following treatment (*Figure 24*).

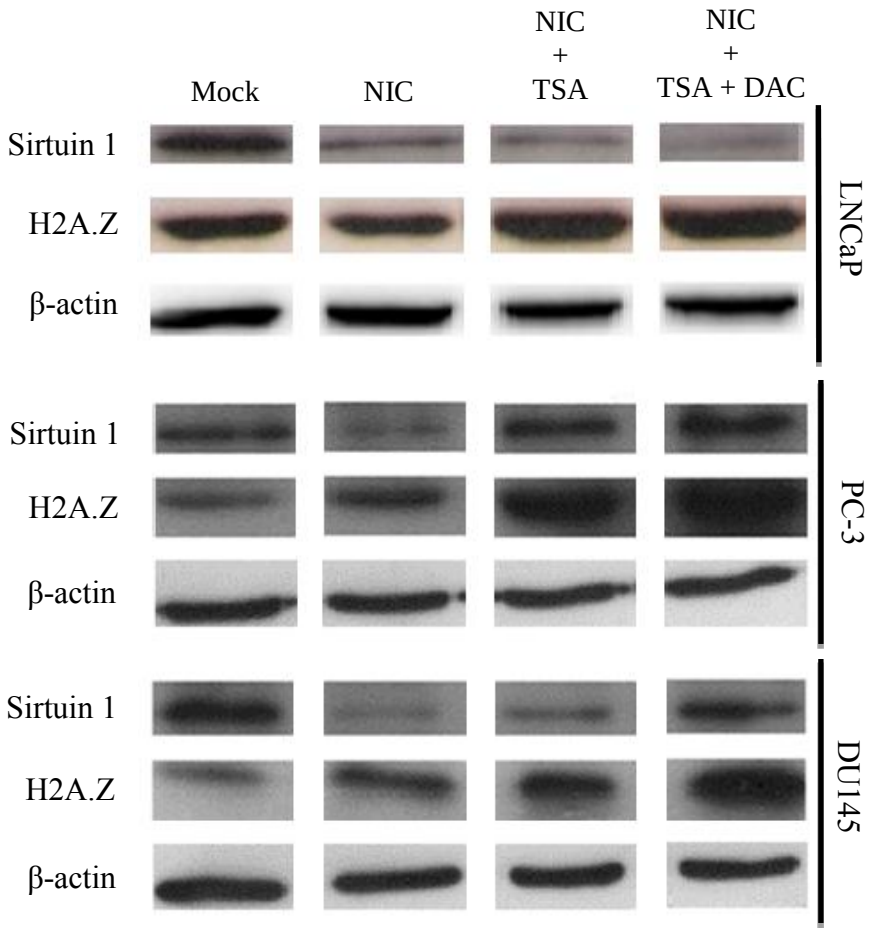

Figure 24 – Western Blot for sirtuin 1, H2A.Z and the constitutive protein β-actin for three prostate cancer cell lines (LNCaP, PC-3 and DU145) after exposure to NIC, alone or combined with epigenetic drugs.

Subsequently, optical density was determined to allow for protein quantity comparison among the different treatment modalities (*Figure 25*).

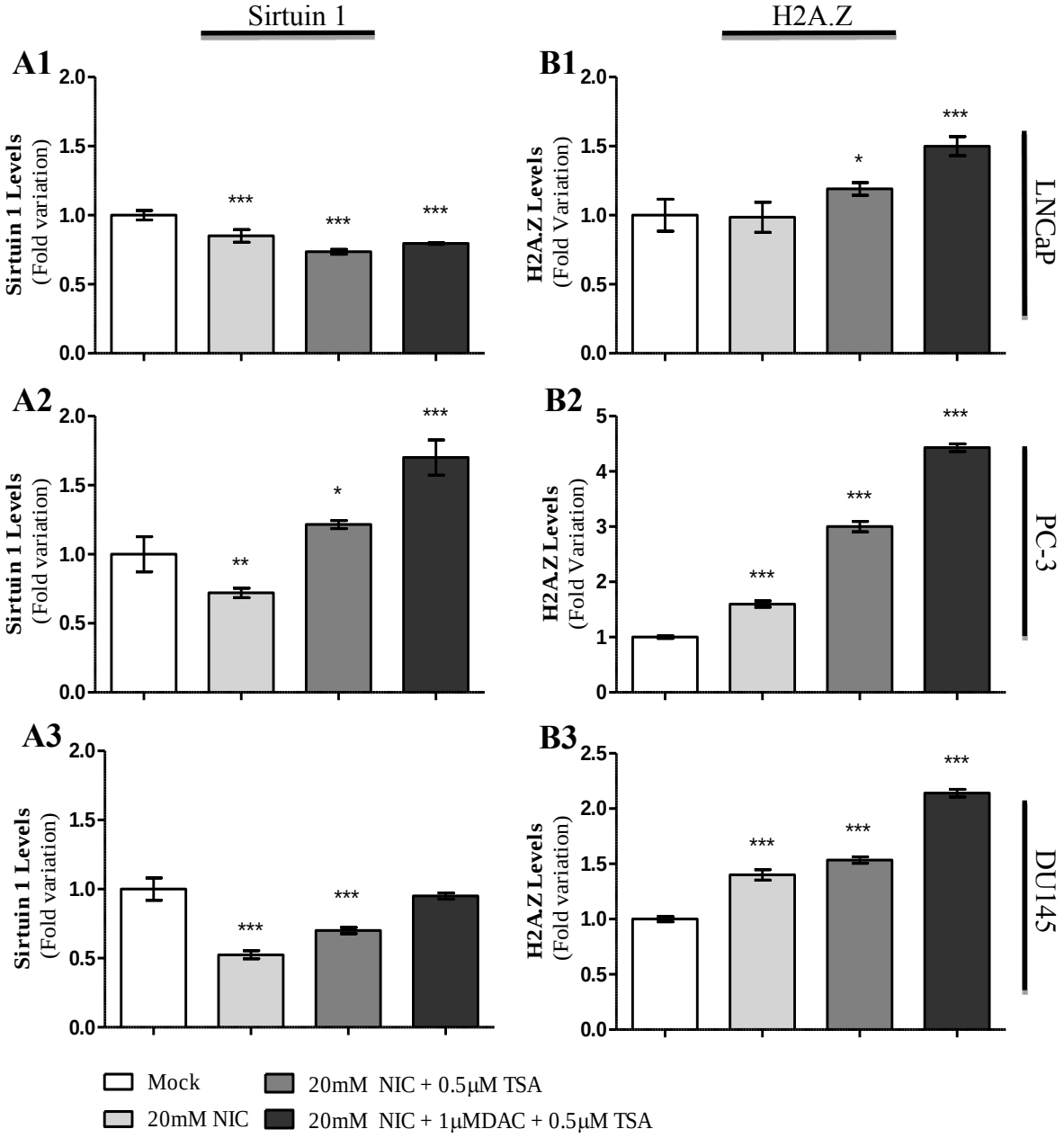

Figure 25 – Optical densities of **(A)** sirtuin 1 and **(B)** H2A.Z for **(1)** LNCaP, **(2)** PC-3 and **(3)** DU145 cell lines after exposition to NIC and/or epigenetic modulating drugs. Results were normalized with β-actin levels and presented as fold variation in comparison with the control experiment.

Dunnett's test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Overall, the three cell lines exposed to NIC showed a decrease in sirtuin 1 levels with a concomitant increase in H2A.Z protein levels (*Figure 24* and *Figure 25*). In LNCaP cells, sirtuin 1 levels decreased after the exposure to NIC, alone or combined with both

epigenetic drugs ($p<0.001$). For DU145 the same was observed but only for cells exposed to NIC alone or combined with TSA ($p<0.001$). Contrarily, in PC-3, sirtuin 1 levels increased after treatment with NIC combined with TSA or DAC plus TSA ($p<0.05$ and $p<0.001$, respectively). Remarkably, PC3 cells exposed to NIC alone showed a statistically significant decrease in sirtuin 1 levels ($p<0.01$).

H2A.Z protein levels increased after inhibition of sirtuin 1 in two of the cell lines (DU145 and PC-3) ($p<0.001$) and a more dramatic effect was disclosed when NIC was combined with TSA ($p<0.05$) or with DAC plus TSA ($p<0.001$).

Hence, the use of NIC alone or combined with TSA, TSA alone or TSA plus DAC, caused a general decrease of sirtuin 1 levels and a simultaneous increase in H2A.Z protein levels.

6. Treatment with resveratrol (RES) is associated with H2A.Z underexpression

Since inhibition of sirtuin 1 was associated with H2A.Z overexpression, we evaluated the opposite effect. Thus, cells were exposed to RES, a selective activator of sirtuin 1, and to epigenetic drugs. Protein levels of both sirtuin 1 and H2A.Z were assessed by Western Blot. As expected, exposure to RES induced a decrease in H2A.Z levels and an increase of sirtuin 1 levels in all PCa cell lines (*Figure 26*).

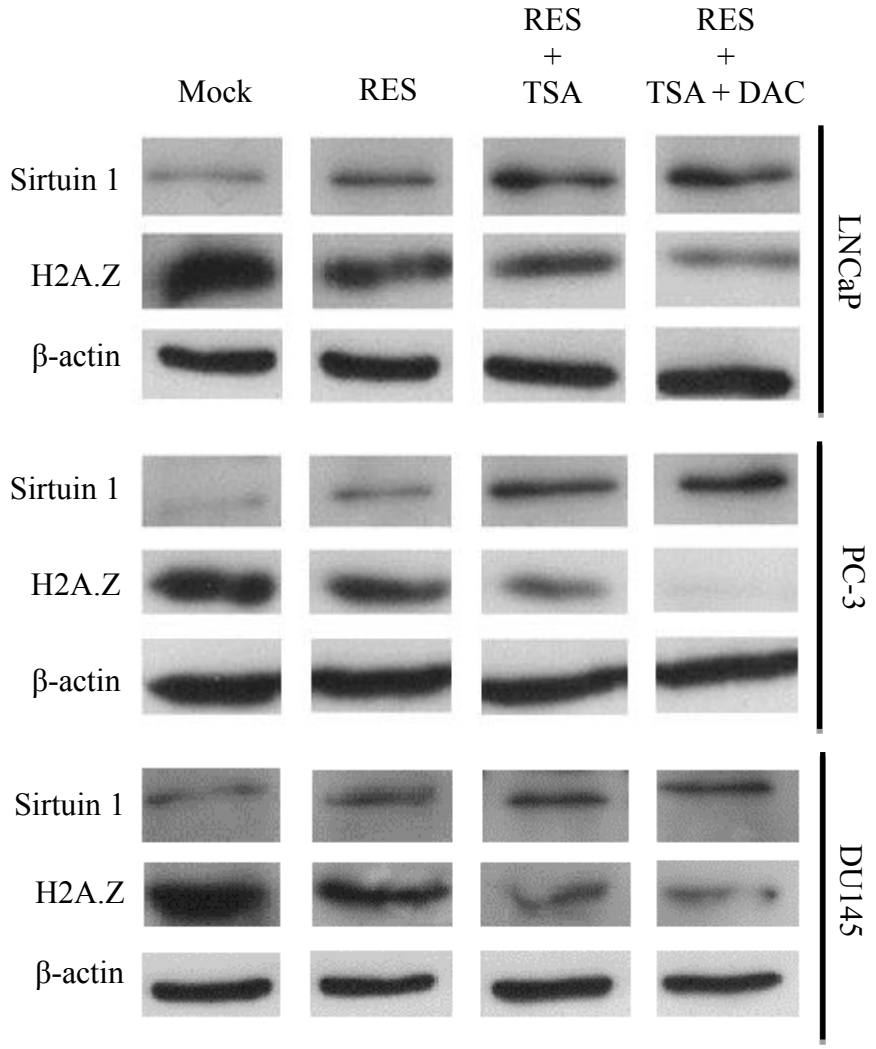

Figure 26 – Western Blot for sirtuin 1, H2A.Z and the constitutive protein β-actin for three prostate cancer cell lines (LNCaP, PC-3 and Du145) after exposition to RES, alone or combined with epigenetic modulating drugs.

Exposure to RES induced lower levels of H2A.Z and higher levels of sirtuin 1 in all PCa cell lines (*Figure 26*). Subsequently, optical density was determined to allow for protein quantity comparison among the different treatment modalities (*Figure 25*).

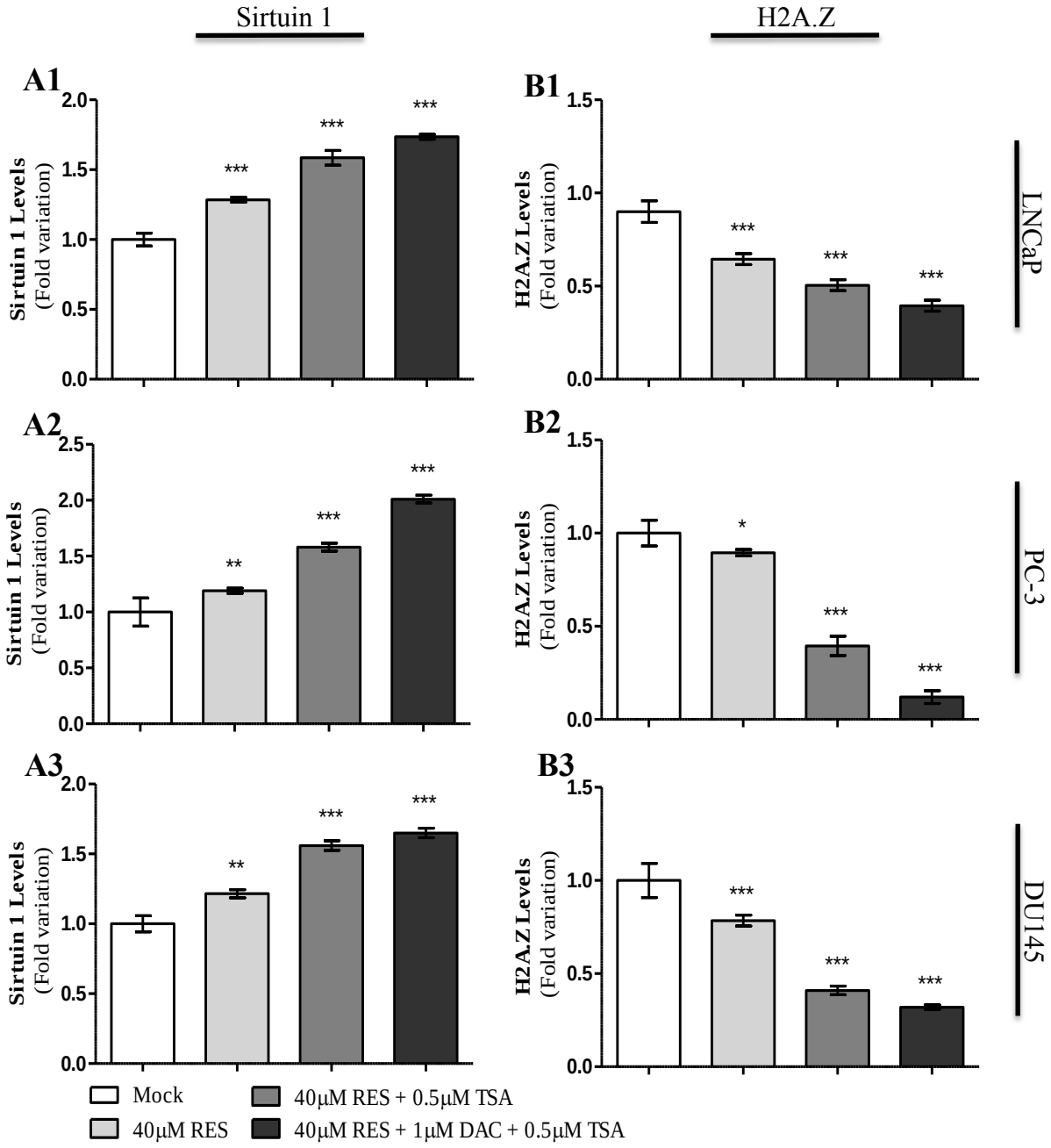

Figure 27 – Optical densities of **(A)** sirtuin 1 and **(2)** H2A.Z for **(1)** LNCaP, **(2)** PC-3 and **(3)** DU145 cell lines after exposition to RES and epigenetic modulating drugs. Results were normalized with β-actin levels and presented as fold variation in comparison with the control experiment.

Dunnett's test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Using optical density analysis, sirtuin 1 protein levels were shown to be significantly increased after PCa cell lines exposure to RES ($p<0.001$ for LNCaP and $p<0.01$ for PC-3 and DU145) (*Figure 27*). This trend was more impressive when

resveratrol was combined with epigenetic modulating drugs ($p<0.001$), and when the sirtuin 1 activator was used simultaneously with DAC and TSA.

The opposite effect was observed for H2A.Z levels: cell lines exposed to RES showed decreased H2A.Z levels ($p<0.05$ for PC-3 and $p<0.001$ for LNCaP and DU145). Interestingly, the lowest levels of H2A.Z were found in PCa cell lines treated with RES in combination with DAC and TSA ($p<0.001$).

Thus, the use of RES alone or combined with TSA or TSA plus DAC, was associated with an overall increase of sirtuin 1 levels, and a concomitant decrease in H2A.Z protein levels.

7. Epigenetic modulating drugs promote the interaction between sirtuin 1 and H2A.Z

In order to validate wether epignetic modulating drugs regulates the interaction between sirtuin 1 and H2A.Z, a crutial step to promote the degradation of the latter, a PLA assay was performed in PC-3 PCa cancer cells (*Figure 28*).

Interestingly, and after exposure to epigenetic modulating drugs, the interaction between sirtuin 1 and H2A.Z abruptly increased, in comparison to the untreated control, *Mock.* Additionally, the results obtained when the combined treatment with DAC and TSA was used were quite similar to those observed after exposure to RES.

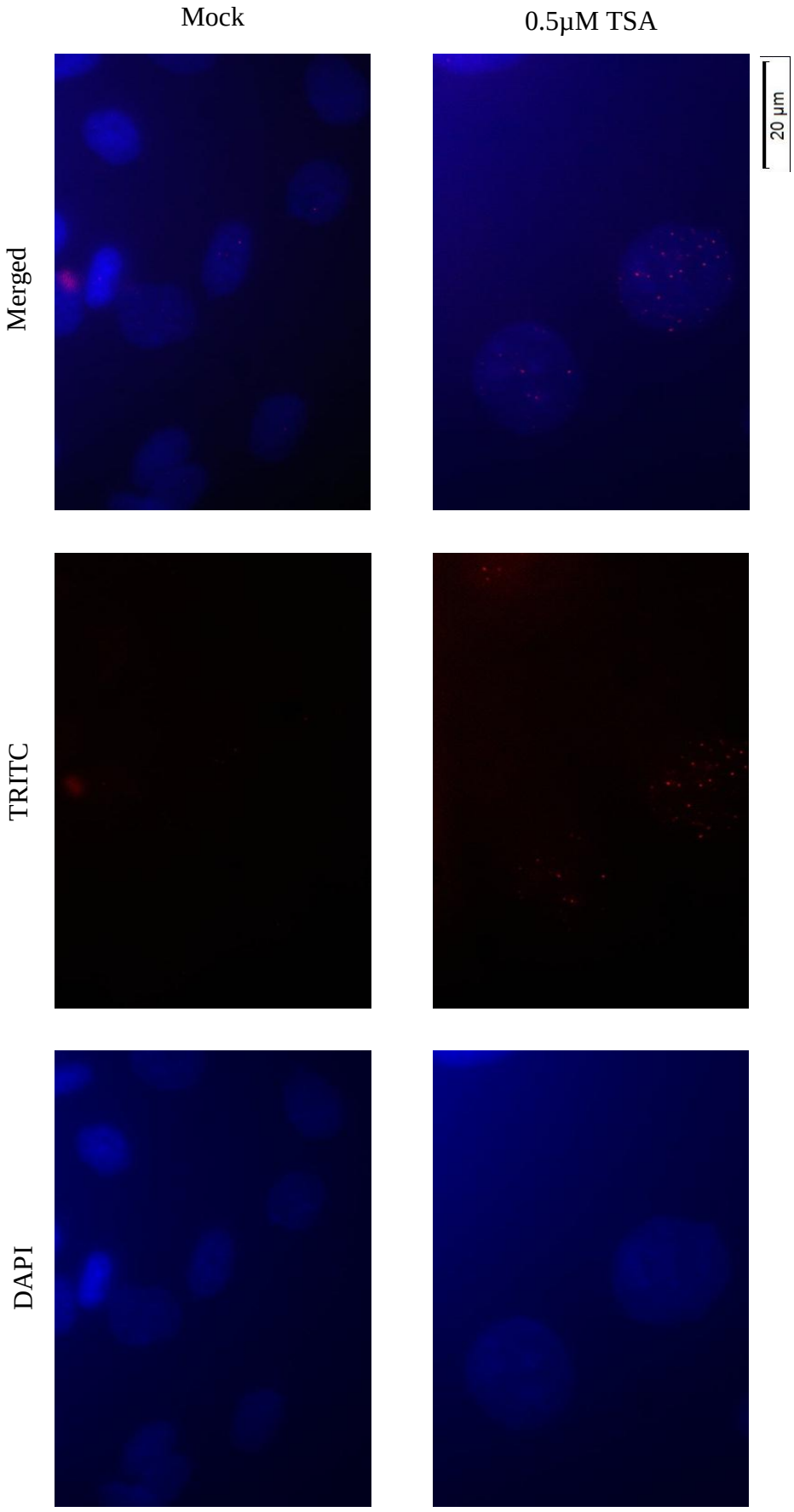

Figure 28 - Legend on the next page.

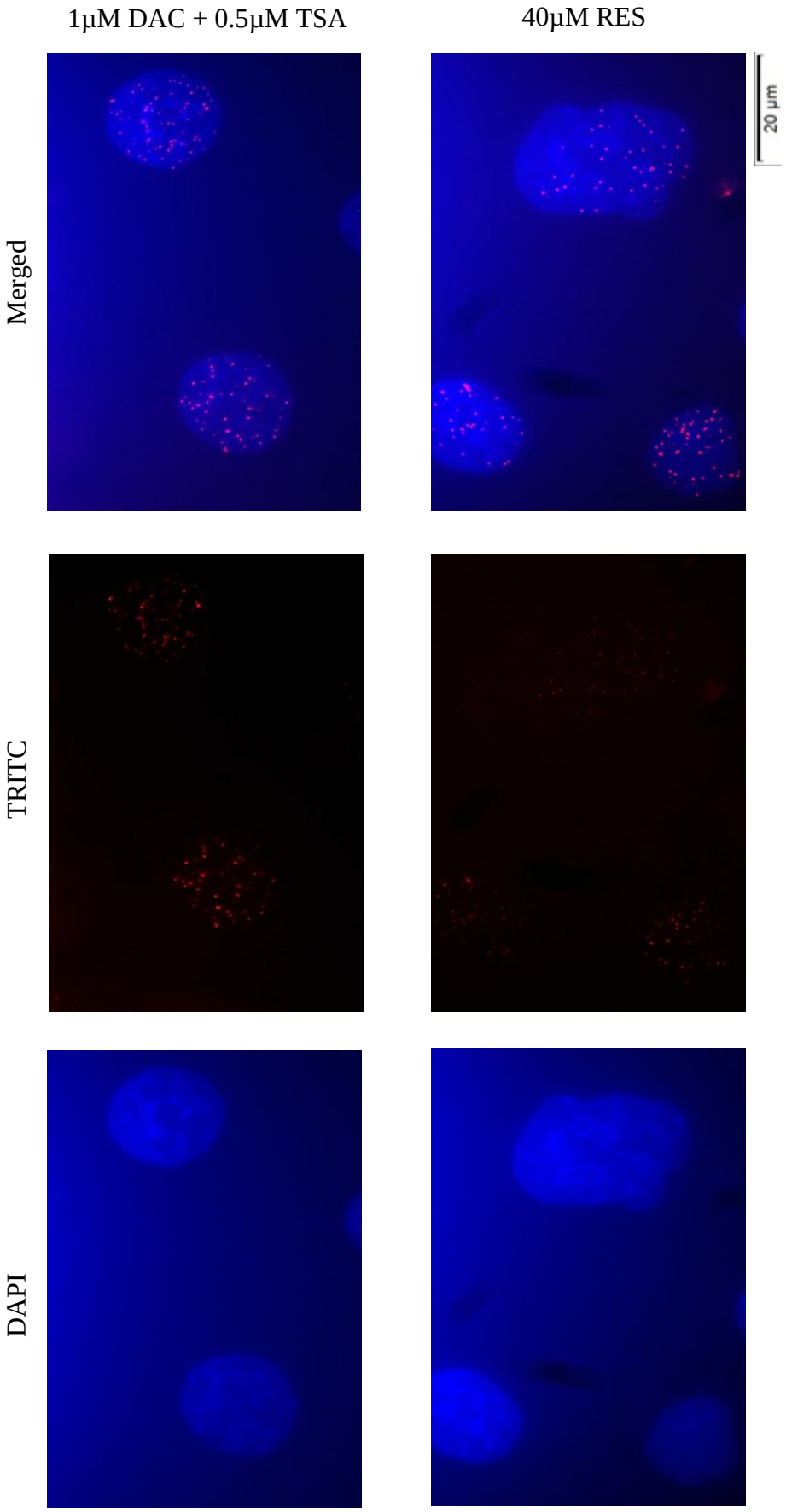

Figure 28 - Detection of sirtuin 1 and H2A.Z interaction in PC-3 cell lines, before and after treatment with epigenetic modulating drugs. PLA signals are shown in red and the nuclei in blue. Magnification: 40x.

8. H2AFZ is overexpressed and SIRT1 is underexpressed in primary prostate cancer tissues

H2AFZ and *SIRT1* transcript levels were assessed in primary tumors and in normal prostatic tissue NPT. Relevant clinical and histopathological data was collected form clinical charts (*Table 8).* No statistically significant differences were found between the age of PCa patients and controls.

Table 8 - Clinical and histopathological features of patients with PCa and normal prostate (NPT).

Clinicopathological Features	**PCa**	**NPT**
Patients, *n*	50	10
Median age, *median (range)*	66 (51 - 74)	63 (45 - 79)
PSA (ng/mL), *median (range)*	9.05 (2.66 - 35.50)	n.a.*
Pathological Stage, *n (%)*		
pT2	18 (36)	n.a.*
pT3	32 (64)	n.a.*
Gleason Score, *n (%)*		
< 7	9 (18)	n.a.*
= 7	36 (72)	n.a.*
> 7	5 (10)	n.a.*

*not available or not applicable

Statistically significant differences in *SIRT1* and *H2AFZ* transcript levels between PCa and NPT samples were found ($p<0.0001$). In fact, tumors displayed a significant underexpression of *SIRT1* and a concomitant overexpression of *H2AFZ* (*Figure 29*).

Statistical analysis did not disclosure any association between *SIRT1* and *H2AFZ* expression levels and the clinicopathological variables of PCa patients.

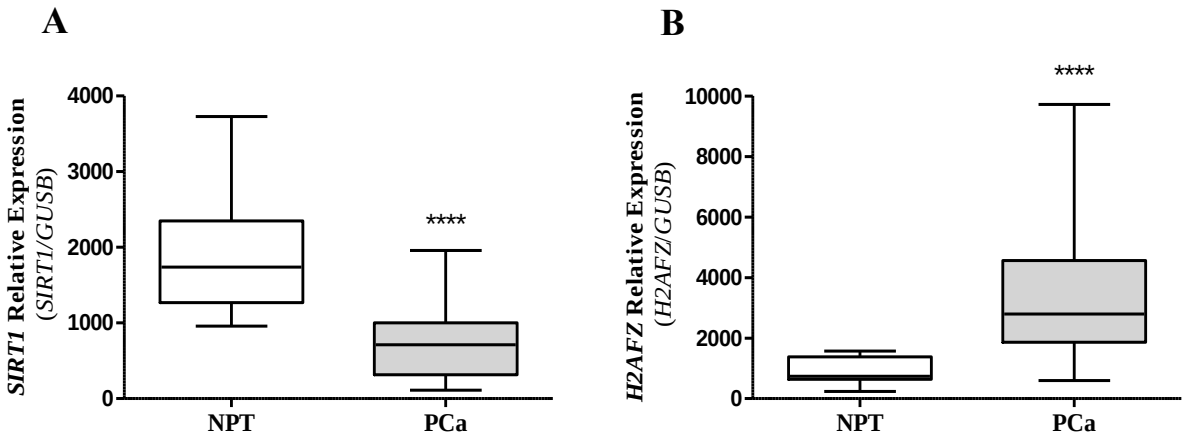

Figure 29 – Distribution of **(A)** *SIRT1* and **(B)** *H2AFZ* relative expression in primary prostate tumor (PCa) and normal prostate tissue (NPT).

Mann-Whitney U-test, **** $p<0.0001$

VI
DISCUSSION

VI – DISCUSSION

It is widely acknowledged that cancer is caused by the accumulation of genetic and epigenetic changes. These alterations result in neoplastic transformation, a process in which neoplastic cells acquire a gene expression profile completely unique and distinct from normal cells [4, 149]. The identification of these profiles has been intensively studied and resulted in a better understanding of tumor biology over the last years. Furthermore, this knowledge might be useful for both cancer diagnosis and prognosis assessment, and might ultimately contribute for a more effective therapeutic approach [106, 150].

Although several studies have reported the effects of epigenetic drugs in some well-known cancer-related genes, mostly tumor suppressor genes [130, 135], there is scarce information concerning the impact of those drugs in expression regulation of genes codifying histone variants, which might influence nucleosome assembly. One of the histone variants that has been studied is H2A.Z. The role of this histone variant in gene transcription regulation is controversial at present, since it has been related both with inactive and active transcription sites of chromatin [151, 152]. Nonetheless, deregulation of this variant have been implicated in several tumor models [50, 153], but not in prostate cancer. Thus, we undertook the task of evaluating the effect of epigenetic modulating drugs on the regulation of H2A.Z expression in PCa.

Firstly, three PCa cell lines were submitted to treatment with DAC and/or TSA, and transcript and protein levels of *H2AFZ* were assessed. We demonstrated that in PCa cell lines, exposure to epigenetic drugs – TSA alone and in combination with DAC – results in increased levels of *H2AFZ* transcript. In fact, the combined treatment with both TSA and DAC induced the highest levels of *H2AFZ* mRNA, advocating a synergistic effect of both drugs, in line with previous reports [154]. Importantly, the increased transcript levels were corroborated by ChIP assay results, which demonstrated an increase in histone activating marks, such as AcH3 and H3K4me2 nearby the TSS. Interestingly, in PCa cell lines treated with epigenetic drugs, AcH2A.Z increased nearby the TSS, contrarily to the total H2A.Z, which remained unchanged. This finding is in accordance in previous reports that have associated this epigenetic mark with gene transcription activation [126], thus justifying the increased levels of *H2AFZ* transcript after PCa cells exposure to TSA. Nevertheless, a

decrease in H2A.Z protein levels was, unexpectedly, observed, suggesting a post-translational mechanism regulating the protein levels of this histone variant.

Accordingly and because sirtuin 1 has been reported to negatively regulate H2A.Z, inducing its degradation in cardiomyocytes [66], we investigated whether epigenetic drugs might also affect *SIRT1* transcription. Indeed, we found that *SIRT1* expression was increased in PCa cell lines exposed to TSA, both at transcript and protein level. Moreover, an enrichment in activating marks (specifically AcH3 and H3K4me2) was observed near the TSS of *SIRT1*, with a concomitant decrease of the repressive mark H3K27me3 [155]. Thus, our results suggest that epigenetic modulating drugs are able to increase the expression of this gene through modifications of the histone marks. Interestingly, levels of AcH2A.Z were rather low and were not altered by the epigenetic treatment. This finding is in accordance with previous studies which have found that, in PCa cell lines, genes not actively transcribed exhibit lower levels of acH2A.Z [126].

To further clarify the putative role of H2A.Z regulation by sirtuin 1 in PCa cell lines, we tested the effect of an inhibitor (NIC) and an activator (RES) of sirtuin 1 on H2A.Z and sirtuin 1 levels [57, 58]. Surprisingly, NIC induced a decrease of sirtuin 1 protein levels. Although unexpected, this has been already reported for leukemic cells [156, 157]. However, our results demonstrate that even in the presence of NIC, exposure to epigenetic drugs is able to increase sirtuin 1 levels. Remarkably, in the latter conditions, H2A.Z protein levels are increased in our *in vitro* model, contrarily to the exposure to epigenetic drugs in the absence of NIC. Thus, although sirtuin 1 levels are increased, NIC is effectively inhibiting sirtuin 1 negative regulation upon H2A.Z through ubiquitin/proteasome-dependent pathway. Conversely, RES alone or combined with epigenetic modulating drugs was associated with decreased H2A.Z protein levels. Interestingly, exposure to RES alone induced an increase in sirtuin 1 protein levels. Although the mechanism can not be derived directly from our data, it has been shown that RES induces *SIRT1* expression [158]. Thus, the combined effect of activation and induced expression of sirtuin 1 by RES justifies the observed decrease in H2A.Z protein levels. Furthermore, exposure to epigenetic drugs just magnifies this effect, mainly through the increased of *SIRT1* gene expression, as expected from the first experiments.

Concerning the mechanism that regulates H2A.Z incorporation into nucleosomes, there is still limited information [159]. However, it is known that the ATP dependent

nucleosome remodeler SNF-2-related CREB-binding protein activator protein (SRCAP) not only inserts H2A.Z into nucleosomes, but is also able to deposit it near gene promoters [159-161]. Remarkably, *SRCAP* has already been described to be overexpressed in PCa [124, 162]. Hence, the enhanced insertion of H2A.Z observed in PCa cell lines might be due to enhanced activity of SRCAP. Nevertheless, H2A.Z can be also incorporated in nucleosomes by other complexes, such as p400 [159]. Although a therapy based on SRCAP repression would seem attractive, it has been reported that total inhibition of H2A.Z insertion into nucleosomes might be pathogenic as well [163, 164]. Hence, a therapeutic approach based on H2A.Z degradation, which would not lead to complete loss of H2A.Z, has been considered more viable and safe [51]. Indeed, our results demonstrate that modulation of H2A.Z levels might be achieved indirectly using epigenetic drugs that are able to induce *SIRT1* upregulation. Moreover, higher levels of active sirtuin 1 might lead to increased deacetylation of Lys-15, triggering a cascade of events that culminates in H2A.Z ubiquitylation on Lys-115 and Lys-121 and, ultimately, in its degradation by the proteosomal pathway [66]. Interestingly, the PLA assay performed in PC-3 cell line showed that, after exposure to epigenetic modulating drugs, the interaction between sirtuin 1 and H2A.Z dramatically increased, suggesting that these drugs were responsible for the promotion of this physical contact, critical for histone variant degradation, as previously reported [66]. It has been also reported that sirtuin 1 not only regulates H2A.Z levels, but also that H2A.Z might influence *SIRT1* expression, existing a reciprocal regulation mechanism between both proteins [66, 165]. Interestingly, our results demonstrate that H2A.Z within nucleosomes neighboring *SIRT1* TSS did not harbor acetylation either before or after treatment with DAC and/or TSA. Thus, we might speculate that H2A.Z negatively regulates *SIRT1* expression, resulting in lower sirtuin 1 levels in PCa cells. This hypothesis is supported by our findings that *SIRT1* transcript levels are lower in PCa tissues compared to normal. There is, however, conflicting data concerning *SIRT1* expression in PCa [64, 65], although it has been recently shown that *SIRT1* is a haploinsufficient tumor suppressor gene [64]. Nevertheless, further studies are required to validate the putative interaction between sirtuin 1 and H2A.Z through colocalization and the physical interaction.

The role of sirtuin 1 in cancer is yet to be fully understood. In fact, *SIRT1* is pointed out as both a tumor suppressor and an oncogene [166]. One of the main features that

validates *SIRT1* as an oncogene is its capacity to inhibit p53 through deacetylation, thus increasing the likelihood of neoplastic transformation [60]. However, it is also known that p53 regulates *SIRT1* transcription through a negative feedback loop. Hence, theoretically, p53 inactivation lowers *SIRT1* activity, which causes increased *TP53* activity [167]. The same occurs for *FOXO3a* [167], *FOXL2* [168], and *E2F1* [169], that might also act as tumor suppressors under certain circumstances. Nevertheless, sirtuin 1 has been also suggested as a tumor suppressor protein, due to its ability to deacetylate the oncogenic β-catenin, inactivating it [170]. Indeed, β-catenin is responsible for the growth of human colon cancer cell lines [170] and, in addition, it has been already reported that BRCA1 interacts with sirtuin 1, enhancing the activity of the latter, reducing growth and increasing apoptosis in cells [171]. Additionally, is has already been reported that sirtuin 1 is responsible for autophagy in PCa cells. Hence, knockdown of this gene induced the formation of PIN, a precursor of PCa, suggesting that *SIRT1* might be, in fact, a tumor suppressor gene in PCa [172].

Herein, we also report for the first time that *H2AFZ* is upregulated in PCa tissues. This finding not only indicates that H2A.Z might play a critical role in prostate carcinogenesis, but also that this protein might be a putative target for advanced PCa therapy, as it has been already proposed for breast cancer [51]. In this vein, the use of epigenetic drugs, that are able to indirectly downregulate H2A.Z through *SIRT1* upregulation, might be an attractive strategy to therapeutically target H2A.Z. Nonetheless, further studies are required to validate *SIRT1* and *H2AFZI* as targets for epigenetic therapy in PCa.

VII
CONCLUSIONS

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The lack of effective therapeutic strategies for castration-resistant PCa have arose the necessity to investigate alternative therapies that might improve treatment of advanced PCa. The role of H2A.Z in carcinogenesis has been already reported in PCa, in which the acetylated form is responsible for oncogenic activation. Nevertheless, the effects of epigenetic drugs on H2A.Z levels have not been previously characterized.

Herein, we exposed PCa cell lines to epigenetic drugs and found an increase in *H2AFZ* transcript levels, associated with augmented levels of histone activating marks near the TSS of *H2AFZ*, as well as a decrease in H2A.Z protein levels after treatment. Owing to the regulatory role of sirtuin 1 on H2A.Z, we further assessed the impact of epigenetic drugs on *SIRT1* at transcript and protein levels. We found that both were elevated and this was associated with enrichment in activating histone marks nearby the TSS of that gene. Furthermore, selective inhibition of sirtuin 1 by nicotinamide resulted in increased H2A.Z protein levels, whereas the reverse effect was observed following treatment with RES, a sirtuin 1 activator. Additionally, exposure of PCa cell lines to epigenetic modulating drugs promoted the interaction between both proteins. We, thus, concluded that H2A.Z levels may be indirectly regulated by epigenetic drugs, through upregulation of *SIRT*1.

Finally, we translated these findings into primary PCa, demonstrating that *SIRT1* was underexpressed compared to normal prostate tissues. Moreover, and for the first time, *H2AFZ* overexpression was verified in PCa. Globally, these results suggest that both genes might be attractive targets for epigenetic therapy in PCa patients.

VIII
FUTURE PERSPECTIVES

VIII – FUTURE PERSPECTIVES

To validate our postulate that sirtuin 1 induces H2A.Z degradation in PCa cells, two complementary studies must be performed:

⇒ Demonstrate colocalization of sirtuin 1 and H2A.Z, by immunofluorescence;

⇒ Assess the interaction between sirtuin 1 and H2A.Z, using a proximity ligation assay, in LNCaP and Du145 PCa cell lines.

To validate both genes as putative target for PCa epigenetic therapy we must assess, in PCa cell lines, the phenotypic impact of:

⇒ Induction of *SIRT1* expression;

⇒ Silencing of *H2AFZ*.

Interestingly, sirtuin1 overexpression will allow the assessment of this effect regarding H2A.Z protein levels.

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